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ON THE POSSIBILITY OF QUANTITATIVE DETERMINATION OF CHROMOPROTEINS BY THE RADIOMETRIC METHOD

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As is known, the iron-containing proteins—the chromoproteins—play an important role in the life processes of the higher animals and man. Some of them, for example, hemoglobin and myoglobin, fulfill the function of transferring oxygen from the lungs to the tissues. Others, such as cytochrome oxidase and the cytochromes, are important catalysts of tissue respiration, fulfilling the role of intermediate electron-carriers.

During the study of the dynamics of oxidative processes in tissues, investigators frequently encounter the need for quantitative determinations of these chromoproteins in biological media. The present methods are not free of faults. Thus, the search for new methods of quantitative determination of the most important chromoproteins, hemoglobin, myoglobin and cytochrome oxidase, is of definite interest. This need motivated the development of a basically new, isotopic method for the quantitative determination of chromoproteins; it is the most sensitive of the extant analytical methods [1-3].

PRINCIPLE OF THE METHOD

The basis of the radiometric method for quantitative determination of some chromoproteins (hemoglobin, myoglobin, cytochrome oxidase) lies in their ability to unite with CO to form the corresponding carboxy compounds [4-6]. Such a reaction of chromoproteins with CO, which is dependent on the presence of divalent iron in the molecule, proceeds in strictly equimolar ratios. By utilizing this property of chromoproteins, their quantitative determination can be accomplished using a radiometric method i.e., by exchanging nonradioactive CO with radioactive $C^{14}O$.

After completly saturating a chromoprotein solution with $C^{14}O$, the radioactivity (A_{total}) will be composed of two values: activity of the $C^{14}O$ chemically united with the chromoprotein (A_{chp}) and the activity of the $C^{14}O$ physically absorbed by the solution ($A_{absorbed}$):

$$A_{total} = A_{chp} + A_{absorbed}. \qquad (I)$$

From this the activity of $C^{14}O$ that is chemically united with the chromoprotein is equal to:

$$A_{chp} = A_{total} - A_{absorbed}. \qquad (IA)$$

From the fact that reaction of $C^{14}O$ with chromoproteins is equimolar, it follows that:

$$A_{chp} = \alpha \cdot C, \qquad (II)$$

where α is the radioactivity per unit of chromoprotein concentration, and C is the chromoprotein concentration.

From Formula (II) it follows that the chromoprotein concentration C in the solution studied can be determined by the following formula:

$$C = \frac{A_{chp}}{\alpha}. \qquad (IIA)$$

By changing the value A_{chp} by its equivalent from formula (IA), the following formula is obtained for the determination of a solution's chromoprotein concentration by the radiometric method:

$$C = \frac{A_{total} - A_{absorbed}}{\alpha}. \qquad (IIB)$$

All of the values necessary for determination of C in this formula can be determined experimentally: A_{total}, by measuring the radioactivity of the chromoprotein solution after its full saturation with $C^{14}O$; $A_{absorbed}$, by determining the radioactivity of the solution (after saturation with $C^{14}O$) in which the iron of the chromoprotein was oxidized to the trivalent form (with sodium nitrate, potassium ferrocyanide and others), for in such a solution $C^{14}O$ will not be united chemically; α can be determined from the formula:

$$\alpha = \frac{A_{total} - A_{absorbed}}{C}, \qquad (IIC)$$

by utilizing for this purpose a standard solution of chromoprotein of known concentration.

It should be noted that during determination of hemoglobin, which is found in the blood in high concentrations, formula (IIB) can be simplified (on the basis of theoretical calculations given below). Since the quantity of physical absorption of CO in water solutions is very low, and with partial atmospheric pressure of CO practically equal to zero, and completely insignificant with the presence of electrolytes in the solution, it should evidently be considered only during determinations of chromoprotein found in solutions in negligibly small concentrations. On the

basis of the above, formula (IIB) can be presented in a simplified form:

$$C = \frac{A_{total}}{\alpha}. \qquad (IID)$$

Quantitative determination of hemoglobin by the radiometric method. The method for quantitative determination of hemoglobin is a special case of quantitative determination of chromoproteins by the radiometric method in general. The method itself, as was shown above, is based on the ability of chromoproteins, in this case, hemoglobin, to react with $C^{14}O$ to give (in equimolar ratios) a stable compound: labeled carboxyhemoglobin ($HbC^{14}O$).

On the basis of the theoretical derivations presented above, formula (IID) was utilized for the determination of blood hemoglobin concentration, where C is the concentration of hemoglobin in gram %, A_{total} is the radioactivity of the blood solution after its complete saturation with $C^{14}O$, and α is the radioactivity corresponding to 1 gram % $HbC^{14}O$. In this way, by knowing the radioactivity of blood following its complete saturation with $C^{14}O$ (A_{total}), and also the radioactivity of 1 gram % $HbC^{14}O$ (α), the percent content of hemoglobin in blood (C) could be determined.

Blood radioactivity could be established by the direct determination of the activity of the blood preparation. In order to determine the radioactivity corresponding to 1 gram % of hemoglobin, blood was used whose hemoglobin content had been determined by the photometric method.

The accuracy of utilizing the simplified formula for determination of the hemoglobin in blood with a blood dilution of 1 : 1 is supported by the following theoretical calculations. As is known, at constant temperature the solubility of a given gas in a given solute is directly proportional to the pressure of this gas above the solute (Henry's law). When a mixture of gases is being dissolved, the solubility of each gas is determined by its partial pressure and is generally equal to the solubility of this gas in a pure state and under a pressure equal to its partial pressure in the mixture. By utilizing the value of the α-coefficient of the physical absorption of the gas by the fluid (water), the quantity of CO physically absorbed by water can be calculated for any given partial CO pressure.

Thus, for example, α_{CO} at 20° is equal to 0.0232 [9]. This means that one volume of water takes up 0.0232 volumes of CO, adjusted to 0° and a pressure of 760 mm of mercury, with a partial pressure of CO above the water of 760 mm Hg. With the content of CO in the atmosphere equal to 0.1%, the solubility of CO in water will be 1000 times less, i.e., $2.32 \cdot 10^{-5}$ ml of CO will be dissolved in 1 ml of water.

In the present experiments we investigate the percentage of physically bound CO compared with that of the chemically bound CO in hemoglobin, when 1:1 diluted blood was taken in 0.1-ml quantities. Normally, this quantity of blood contains, on the average, ~ 7.5 mg of hemoglobin, which binds 10.5 μliter CO (1 mg binds 1.34 μliter CO [6, 8], and the physically bound CO in the water, when the air content of the water is 0.1%, will be 0.00232 μliter, which, in relation to the CO chemically bound by hemoglobin, is only 0.022%. Taking into account the fact that the CO content in the surrounding air is practically equal to zero, and the physical absorption of gases is lowered by the presence of electrolytes in the solution, the value for physical absorption of CO can be disregarded.

METHODS

Equipment and reagents.

In order to conduct the investigation, the following equipment and reagents were necessary: a radiometer of type V-2; block counter MST-17, placed in a lead housing SD-2, or any other lead shielding; a target with surface diameter of 18 mm; a microgasometer with a volume of 500 ml; radioactive carbon monoxide ($C^{14}O$)* with specific activity not lower than 12.4 μC/ml (preparations with higher specific activity can be utilized).

The carbon monoxide ($C^{14}O$) was rarified five-fold with air in the gasometer, and a $C^{14}O$–air mixture was obtained with specific activity of 2.48 μC/ml; this mixture was used for the saturation of blood solutions. The stabilizing mixture was of the following composition: Sodium oxalate-400 mg, sodium hydrosulfite-1000 mg, saponin-400 mg. All of the components of the mixture should be finely ground in a mortar and stored in a closed flask. The stabilizing solution was prepared from this mixture according to need (18 mg of mixture per 1 ml of water) and was not stored for longer than a day.

Course of determination.

Determination of radioactivity in 1 gram% $HbC^{14}O$ (α_{mean}). In order to determine the percent of hemoglobin in blood by the photometric method, 10 ml of 0.04% ammonium solution and 0.1 ml of blood taken from a finger were poured into a tube; to these was added several mg of sodium hydrosulfite; the hemoglobin content was then determined by the photometric method [18].†

For the determination of the radioactivity of the blood preparation (A_{total}), 0.2 ml of the stabilizing solution and 0.2 ml of blood which was taken from the finger of the same invalid were poured into a small tube (inner diameter 8 mm). The stabilizing solution kept the blood from clotting, assisted in hemolysis, and also changed

*Radioactive carbon monoxide was obtained from radioactive formic acid [7].

†Measurement was conducted with a green filter; to determine the coefficient by which extinction, obtained with the green filter, must be multiplied, blood was used, the hemoglobin content of which was determined spectrophotometrically in the SF-4 apparatus by co-worker N. D. Kuznetzova (SKTB "Biophys. Apparatus"), to whom the authors express their gratitude.

TABLE 1. Data for the Computation of $\alpha_{average}$ from Six Samples of Blood

Avge. activity cpm and absolute error of determination (Δ)*	Relative error of determination, %† (δ)	Hemoglobin concent., g%	Value of α	Average arithmetic value of α from 6 samples
419±12	±2,8	14,9	28,1	
503±13	±2,5	15,7	32	
419±12	±2,8	14,7	28,5	
436±11	±2,5	15,1	28,8	29,1
438±12	±2,7	15,3	28,6	
398±11	±2,7	13,9	28,6	

*Absolute error of determination (Δ) was determined according to the formula

$$\Delta = \sqrt{\frac{n}{t} + \frac{n\phi}{t\phi}}.$$

†Relative error of determination (δ) was determined according to the formula

$$\delta = \frac{\Delta \cdot 100}{n - n\phi}$$

[1] where n is the rate of count of the preparation and the background in cpm; t is the time of determination, $n\phi$-background count in cpm.

oxyhemoglobin into reduced hemoglobin and prevented its further union with atmospheric oxygen.

Blood prepared in this way was saturated (in the tube) with $C^{14}O$, by means of a capillary tube connected to the gasometer. The saturation was accomplished by slow passage of small bubbles of the $C^{14}O$–air mixture through the blood mixture for 2 min.‡ Saturated blood in 0.1-ml quantities was transferred onto the planchet, spread evenly with a glass rod over the planchet surface, and immediately, using the block counter (MST-17**), which was connected to apparatus V-2, the radioactivity of the fluid blood preparation was determined for 3 min. This duration of activity measurement was taken from the calculation of the relative error of measurement of ±3% [1]. From the measurement data of three preparations an average value for blood radioactivity (A_{total}) was obtained. Knowing A_{total} and C, the value α for blood was obtained by means of formula (IID).

Properties of α theoretically should be the same for hemoglobin of various animals and man, for it is one single quantity of hemoglobin that binds one single quantity of CO, namely, 1.34 ml of CO for every 1 gram of hemoglobin. However, during dilution of blood 1:1 it was observed that the values of α experimentally obtained for blood preparations of various individuals (humans) were somewhat different from one another, which evidently may be explained by technical error. In connection with possible technical error during determination of even parallel samples, an average calculated value of α for several blood preparations taken from different people was used for the purpose of leveling off the errors. The value of α was obtained arithmetically from six average samples of blood (Table 1).

Determination of hemoglobin. For the determination of blood hemoglobin by the radiometric method, the preparations were prepared by the method described above, and then their activity was determined. From determination data of three preparations the average blood activity (A) was established. Knowing A and the average activity of 1 gram % of hemoglobin ($\alpha_{average}$), the percent content of hemoglobin in blood was determined according to formula (II D):

$$C = \frac{A}{\alpha_{average}}.$$

For example, if the average activity of blood preparation was equal to 389 cpm and $\alpha_{average}$ was equal to

‡Preliminary experiments have shown that complete blood saturation with $C^{14}O$ takes place in one minute. The radioactivity of blood preparations saturated with $C^{14}O$ for one and two minutes is the same.

**It should be noted that during radioactivity determinations of fluid blood preparations with the block counter, a lowering of the radioactivity of preparations due to loss of $C^{14}O$ from the surface layers of the solution occurred with time. However, the errors related to this were small in the measurement of the radioactivity of blood preparations, which was used for the determination of α and for unknown blood preparations. Such measurements were carried out under identical conditions (exactly 30 sec after placing on the planchet, and for 3.0 min).

TABLE 2. Blood Hemoglobin Content According to Determination Data from Radiometric and Photometric Methods

Aver. activity of preparation and absolute error of measurement in cpm (Δ)	Relative error of measurement, % (δ)	Determination of hemoglobin content		Error of the radiometric method in relation to the photometric, %
		Radiometric method	Photometric method	
488±13	±2,6	16,8	15,9	+6
422±12	±2,8	14,5	14,3	+1
466±12	±2,5	16,0	15,7	+2
461±12	±2,6	15,8	15,3	+3
389±11	±2,7	13,4	13,3	+1
394±11	±2,7	13,5	13,7	−1
441±12	±2,7	15,2	15,7	−3
400±11	±2,7	13,3	14,3	−3
422±12	±2,8	14,5	15,2	−5
393±11	±2,7	13,5	14,4	−6
462±12	±2,5	15,9	15,5	+3
441±12	±2,7	15,2	14,5	+5
467±12	±2,5	16,0	16,3	−2
457±12	±2,6	15,7	15,1	+4
447±12	±2,6	15,4	16,1	−4
444±12	±2,7	15,3	14,6	+5
482±13	±2,6	16,5	15,5	+6
443±12	±2,7	15,2	15,3	−1
511±13	±2,5	17,5	16,9	+4
450±12	±2,6	15,5	15,1	+3
445±12	±2,6	15,3	16,1	−3
458±12	±2,6	15,7	15,3	+3
464±12	±2,6	15,9	15,9	—
448±12	±2,6	15,4	15,4	—

29.1 cpm, then the hemoglobin content of blood (C) was equal to:

$$C = \frac{389}{29.1} = 13.4 \text{ gram \%}.$$

RESULTS AND DISCUSSION

The method described was utilized for the determination of blood hemoglobin content of schizophrenics and neurotics (total of 24 men) and a comparison was made between data obtained by the radiometric and photometric methods; the data from the photometric method was taken as 100% (Table 2). Results of hemoglobin determinations by the radiometric method compared favorably with data from the photometric method. In this way, by allowing the relative error of radioactivity determination to be ± 3%, the possibility of determining blood hemoglobin by the radiometric method with an accuracy of up to ± 5-6% in relation to the photometric method arose.

On the basis of literature data [1-3], it can be counted on that the described radiometric method for hemoglobin determination could be substantially improved by using scintillation counters, which possess high effectiveness of counting. Utilization of a scintillation counter will permit achievement of high statistical reliability of measurement when preparations of $C^{14}O$ with low specific activity are utilized during shorter intervals of measurement. Also, the obtained data for the quantitative determination of blood hemoglobin show that the proposed method can be utilized in practice in those cases in which the utilization of spectroscopic or colorimetric methods is made difficult or impossible due to the presence in the substrates studied of other pigmented substances, for example, during the investigation of pus, urine, etc. The utilization of the proposed principle of quantitative determination by the radiometric method of such iron-containing chromoproteins as myoglobin and cytochrome oxidase is especially promising. The utilization of the radiometric method is interesting for the quantitative determination of cytochrome oxidase, for at the present time there are no other methods for quantitative determination of this enzyme. However, for the quantitative determination of myoglobin and of cytochrome oxidase, especially the latter, there is a need for increase in the sensitivity of the method by several thousand times, which is fully possible only in the presence of $C^{14}O$ preparations with high specific activity (1-2 μC/ml) and with utilization of counters into which the preparation to be measured is intorduced as a gas, or counters such as the 4π scintillation counter [3, 10]. In addition, the proposed radiometric method for quantitative determination of chromoproteins evidently could be utilized for quantitative determination of derived chromoproteins such as methemoglobin of blood, met-myoglobin in muscles, and likewise, the oxidized and reduced forms of cytochrome oxidized and reduced forms of cytochrome oxidase in tissues.

Using blood hemoglobin as a example, let us investigate the possibility of determining the oxidized forms of chromoproteins (methemoglobin). The principle of the radiometric method for quantitative determination of methemoglobin is a particular case of the above-proposed principle for the quantitative determination by radiometric methodsof chromoproteins in general. In accordance with this principle, the blood radioactivity following complete saturation with $C^{14}O$ is directly proportional to its content of radioactive hydroxyhemoglobin ($HbC^{14}O$). From this it follows that in blood not containing methemoglobin, all of the hemoglobin (100%) is capable of uniting with $C^{14}O$ to form $HbC^{14}O$. Activity of such blood is equal to $A_1 = \alpha \cdot 100$, where A_1 is the activity of blood not containing MtHb; α is the activity corresponding to 1% $HbC^{14}O$; 100 is the percent content of $HbC^{14}O$.

During oxidation of a portion of blood hemoglobin to methemoglobin (for example, X % hemoglobin oxidized to methemoglobin), only that portion of hemoglobin which was not oxidized to methemoglobin will be united with $C^{14}O$, namely, (100 - X)% of hemoglobin. The activity of such blood will be equal to:

$$A_2 = \alpha \cdot (100-X).$$

where A_2 is the activity of blood containing X% MtHb; α is the activity corresponding to 1% $HbC^{14}O$; (100-X) is the percent of hemoglobin united with $C^{14}O$. From this, knowing A_1 and A_2, the percent content of blood methemoglobin (X) can be derived:

$$\frac{A_1}{A_2} = \frac{\alpha \cdot 100}{\alpha \cdot (100-X)}, \text{ from which } X = 100\,\frac{A_1 - A_2}{A_1}.$$

In this way the quantitative determination of methemoglobin reduces to the finding of A_1 and A_2, i. e., the activity of two preparations, one of which does not contain methemoglobin, and the second of which does.††

A further problem is the utilization of the proposed principle for the determination of other chromoproteins (myoglobin and cytochrome oxidase) and their metcompounds.

SUMMARY

With the help of the principle proposed by the authors, based on the ability of chromoproteins to unite with radioactive carbon monoxide ($C^{14}O$) in equimolar ratios, quantitative determination of blood hemoglobin is made possible.

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††It is possible to obtain preparation A_1 from blood containing methemoglobin by reducing it with sodium hydrosulfite and saturating with $C^{14}O$. Preparation A_2 is obtained by saturating blood containing methemoglobin with $C^{14}O$.

ON THE KINETICS OF HYDROGEN PEROXIDE DECOMPOSITION BY CATALASE

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On the basis of careful experimental investigation of the kinetics of enzymatic H_2O_2 decomposition, Ivanova [1] and Ogura [2] came to the conclusion that the dependence of the reaction rate, V, on the concentration of catalase, F, and on the concentration of hydrogen peroxide, C, was best expressed by the Michaelis-Menten equation:

$$V_0 = rF_0\frac{KC_0}{1+KC_0}, \qquad (I)$$

where r and K are constants.

However, using the Michaelis-Menten equation, we can compute the reaction rate of H_2O_2 decomposition only for the initial moment, t = 0, but we cannot predict the changes which occur as the enzymatic process proceeds: The enzyme undergoes inactivation and its active concentration is continually lowered; this is not taken into account in equation (I). With small catalase concentration and large concentrations of H_2O_2, the decomposition reaction of the latter does not proceed to the end due to complete enzyme inactivation, i.e., the reaction rate V becomes equal to zero when C > 0, because the active catalase concentration F is concurrently lowered to zero.

In order to determine the relation between the change in active catalase concentration and the changes in H_2O_2 concentration during the course of the enzymatic process, the authors conducted an experimental study of the catalytic capacity of a catalase preparation over wide ranges of enzyme and substrate concentrations at various temperatures and pH values of the reaction medium and with addition of an enzyme poison. The value for catalytic capacity was expressed in millimoles of H_2O_2 which under the chosen experimental conditions, were decomposed by one milliliter of catalase preparation until its complete inactivation.

In this work the catalytic capacity of blood catalase was also studied.

EXPERIMENTAL AND DISCUSSION

Determination of catalase catalytic capacity. A catalase preparation was obtained from horse liver by the method described by Euler and Josephson [3] and was purified by the method of Tsuchihashi [4]. In order to maintain hydrogen-ion concentration at a constant level, the catalase preparation and H_2O_2 were diluted to the necessary concentrations with phosphate buffer (1/15 (1/15 M, pH 6.8).

For each experiment, concentrations and volume relations of H_2O_2 and catalase were chosen such that, as the result of the solution's reaction, there would take place complete catalase inactivation while in the reaction mixture there would be undecomposed H_2O_2.

Into a water thermostat held at 37°, a flask with 45 ml of H_2O_2 solution and a test tube with catalase solution were placed. After 10 min of warming, 5 ml of catalase solution was added to the flask and timing was initiated with a stopwatch. Changes in H_2O_2 concentration were determined by titration of samples with potassium permanganate; the samples were taken every 5-10 min. The taking of samples was discontinued when the last three samples showed a constant concentration of H_2O_2; this was indicative of catalase inactivation. The time interval for complete catalase inactivation, t_k, was determined according to the curve (Fig. 1) constructed on the basis of the experimental data.

The difference between C_0 and C_k concentrations was utilized for calculation of the total quantity (in millimoles) of the H_2O_2 decomposed during the time of the experiment in 50 ml of reaction mixture $(C_0-C_k)\cdot 50$, and subsequently for the calculation of the catalytic capacity E according to the formula:

$$E = \frac{(C_0 - C_k)\cdot 50}{5/n},$$

where n is the value showing the dilution of the catalase preparation prior to the experiment. Concurrently with the principal experiment, and under the same conditions, two control experiments were conducted: H_2O_2 without catalase and catalase without H_2O_2. In the first experiment, we determined the extent of spontaneous hydrogen peroxide decomposition CP (in percent), according to the equation:

$$CP = \frac{(C_0 - C_1)\cdot 100}{C_0},$$

where C_1 is the H_2O_2 concentration after t_k minutes.

TABLE 1. Catalytic Capacity of a Catalase Preparation under Various Conditions. Where specific temperature and pH are not indicated, they correspond to 37° and 6.8, respectively.

Series	Conditions of expt.	mM/ml	mM/ml	t_k, min	E, mM H_2O_2/ml of catalase preparation	CP, %	CI, %
	Experiments with a catalase preparation						
1	Preparation 1:100, various H_2O concentrations	0,233	0,122	25	111		
		0,478	0,370	15	108		
		0,720	0,604	12	116		
		1,464	1,351	10	113		
2	Preparation dilutions						
	1 : 50	0,233	0,022	35	105,5		
	1 : 100	0,233	0,122	25	111		
	1 : 150	0,210	0,134	19	114		
	1 : 200	0,226	0,166	15	120		
	1 : 300	0,221	0,180	7	123		
	1 : 400	0,217	0,187	5	120		
3	Preparation 1:200, temperature						
	25°	0,241	0,138	50	206	1,20	1,6
	37°	0,226	0,166	15	120	1,30	3,8
	50°	0,230	0,196	5	68	4,1	7,6
4	Preparation 1:200 pH:						
	4,97	0,231	0,182	20	98	—	—
	6,8	0,226	0,166	15	120	—	—
	8,97	0,235	0,185	19	100	—	—
5	Preparation 1:100, inactivator						
	NaF 3%	0,234	0,138	40	96	—	—
	$HgCl_2$ 0,01%	0,238	0,183	30	55	—	—
	» 0,1%	0,238	0,196	25	42	—	—
	» 1%			Complete inactivation			
	Without activator	0,233	0,122	25	111	—	—
	Experiments with blood						
6	Subject N Blood dilution 1 : 1000	0,0545	0,0230	20	315	—	—
	Various H_2O_2 concentrations	0,1090	0,0770	12	320	—	—
	Subject N Blood dilution						
	1 : 1000	0,0545	0,023	20	315	—	—
	1 : 2000	0,0545	0,038	15	330	—	—
	Subject B Blood dilution						
	1 : 1000	0,049	0,020	25	290	—	—
	1 : 2000	0,049	0,033	18	320	—	—
7	Subject N Blood dilution						
	1 : 1000	0,059	0,029	18	300	—	—
	1 : 500	0,059	0,001	32	290	—	—

Note. Values of E presented in the Table were established by numerous repetitions of the experiment under chosen conditions.

The second experiment consisted of placing into the thermostat, together with the reaction flask, two test tubes, one of which contained 9 ml of the phosphate buffer and the other containing 1 ml of catalase solution of the same concentration as in the main experiment. After 10 min, to one of the test tubes we added 10 ml of 0.1 N H_2O_2 and then, after 5 min, 2.5 ml of 50% H_2SO_4; the contents were then titrated. At the end of the main experiment, the same procedure was followed on the catalase solution in the other test tube. From the lowering of the rate of H_2O_2 decomposition, the value for spontaneous catalase inactivation (CI) was calculated (in percent), according to the formula: $CI = (V_1 - V_2) \cdot 100 / V_1$, where V_1 is the rate of H_2O_2 decomposition in the first test tube and V_2 is that in the second.

The blood catalytic capacity of a human subject under study was determined by an analogous method. The blood was taken by a common method in 0.02-ml

quantities and was diluted with distilled water prior to the experiment. Experiments were conducted with various concentrations of blood and of H_2O_2 in the reaction mixture.

Experimental data concerning catalytic capacity of catalase. Results of experiments with catalase preparation are presented in Table 1. In all of the experiments conducted at 37°, the value for spontaneous H_2O_2 decomposition varied between 1 and 1.5%, and the value for spontaneous catalase inactivation, from 2 to 4%. Both values increased noticeably with an increase in temperature to 50° (Table 1, series 3), but did not introduce essential corrections into the main experiments.

In some experiments with blood catalase, H_2O_2 and blood were diluted with phosphate buffer (1/15 M, pH 6.8) or, in other experiments, with distilled water, as was recommended in the method of Bakh and Zubkova [5]. The absence of buffer in the reaction mixture was almost without influence on the obtained results.

The experimental data obtained show that catalytic capacity and, therefore, catalase inactivation were practically independent of H_2O_2 concentration (Table 1, series 1). The reduction of the time intervale for full inactivation (t_{ik}) with the increase in H_2O_2 concentration pointed only to the increase in the rate with which one milliliter of the prepared catalase preparation, in each experiment, decomposed 108-116 millimoles of H_2O_2. With dilution of the catalase preparation (small dilutions), its catalytic capacity increased somewhat in the beginning and then became practically unchanged (Table 1, series 2).

Temperature exerted a sharp influence on the catalytic capacity of the catalase preparation; with lowering of the temperature, the catalytic capacity increased sharply (Table 1, series 3). At 0° it appeared to be so large that it could not be determined experimentally. Concentration of H_2O_2 in this case did not settle at a constant level even after one day. The hydrogen indicator (pH) likewise expressed an essential influence on the catalytic capacity of a catalase preparation. At pH values close to seven, both the catalytic capacity and the rate of H_2O_2 decomposition were the highest (Table 1, series 4). Catalase inactivators lowered not only the rate of H_2O_2 decomposition, but also the enzyme catalytic capacity (Table 1, series 5).

The constancy of the catalytic capacity values with changes in substrate and enzyme concentrations was also observed in the experiments with blood (Table 1, series 6 and 7). This fact attested to the regularity of conversion of an active part of catalase into an inactive form during the process of enzymatic decomposition of H_2O_2. The nature of this regular conversion is yet unknown. Interest is presented in the fact established in the study that the catalytic capacity of catalase was reduced by an increase in temperature, which by itself inactivates the enzyme. It follows that temperature and the enzymatic process affect catalase activity in the same manner.

The simplicity and comparatively high accuracy of the experimental determination of catalytic capacity give the basis for considering this value to be a quite reliable indicator of catalase activity. Thus, the data obtained once again confirm the reliability of the well-known method of Bakh and Zubkova for the determination of blood catalase activity.

As is shown by data in Table 1 (series 7), the enzymatic reaction was essentially ended before 30 min. Only after a two-fold increase of blood concentration in the reaction mixture, as compared with blood concentration in the method of Bakh and Zubkova, was there complete catalase inactivation in 32 min (Table 1, series 7). Consequently, the 30-min incubation chosen by Bakh and Zubkova assured maximal decomposition of H_2O_2 by the experimental blood sample.

Derivation of the kinetic equation. The data obtained concerning the catalytic capacity of the catalase preparation point to the close relation between changes of H_2O_2 concentration and the active enzyme concentration; a unit of catalase mass at a given temperature and at a given pH value of the reaction mixture decomposes a definite amount of H_2O_2, which is practically independent of enzyme and substrate concentration, i.e., $\left(\frac{\delta C}{\delta F}\right)_{T,pH} = E$, where E is the catalytic capacity of catalase.

From this, by integration, we obtain the equation for the decrease in active enzyme concentration F during the enzymatic process with the extent of lowering in H_2O_2 concentration in the reaction mixture: $F = F_0(C_0 - C)/E$, where F_0 and C_0 are respectively the initial concentrations of catalase and H_2O_2. Substituting the value of F into the Michaelis-Menten equation (I):

$$V = r\left(F_0 - \frac{C_0 - C}{E}\right)\cdot\frac{KC}{1+KC}, \qquad \text{(II)}$$

$$V = r\left(F_0 - \frac{C_0}{E} + \frac{C}{E}\right)\cdot\frac{KC}{1+KC}. \qquad \text{(III)}$$

Equation (II) shows the change in the proceeding rate V with the lowering of H_2O_2 concentration as a result of its enzymatic decomposition. The dependence of the initial rate V_0 on the concentration C_0 will be expressed by the Michaelis-Menten equation. Actually, at the initial moment, $C = C_0$ and in this case equation (II) converts to equation (I).

For the purpose of practical utilization of equations (II) and (III), it will be arranged to express the enzyme concentration F_0 in units of: milliliters of catalase preparation in one milliliter of the reaction mixture,* and

* If 50 ml of the reaction mixture contains 5 ml of catalase preparation, previously diluted 100 times, then $F_0 = 5/(100)(50) = 0.001$ ml preparation / ml of reaction mixture.

the catalytic capacity E by the number of millimoles of H_2O_2 decomposed by one milliliter of catalase preparation.

The ratio C_0/E in equation (III) represents that theoretical catalase concentration F_e in W ml of reaction mixture with which there should take place concurrent complete decomposition of H_2O_2 and complete inactivation of catalase.

Actually, 1 ml of catalase preparation decomposes E millimoles of H_2O_2 and F_eW milliliters of catalase preparation should decompose C_0W millimoles of H_2O_2; from this, $F_e = C_0/E$.

Substituting F_e into equation (III), we obtain:

$$V = r\left(F_0 - F_e + \frac{C}{E}\right)\frac{KC}{1+KC}. \qquad \text{(IV)}$$

At F_0-Fe < 0 there is incomplete decomposition of H_2O_2; the rate V becomes equal to zero when the term $F_0 - F_e + C/E = 0$; i.e., the enzymatic process ends when $C = (F_e - F_0)E = (C_0 - F_0E)$ as the result of complete catalase inactivation. At $F_0 - F_e > 0$ there will take place complete decomposition of H_2O_2, but in the reaction mixture there will still remain active catalase, the concentration of which can be easily found by the formula:

$$F = F_0 - F_e = F_0 - \frac{C_0}{E}.$$

If $F_0 = F_e$ then, as was shown earlier, the enzymatic process ends and then there will take place complete decomposition of H_2O_2 and complete inactivation of catalase. In this case equation (IV) converts into the equation:

$$V = \frac{r}{E}\cdot\frac{KC^2}{1+KC}. \qquad \text{(V)}$$

At $F_0 - F_e = 1/KE$ equation (IV) converts into the equation

$$V = \frac{r}{E}\cdot C. \qquad \text{(VI)}$$

By integrating equation (IV) ($\int_0^t$) with the conditions $F_0 \neq F_e$, 0 and $E \neq \infty$, there is obtained the equation:

$$t = \frac{1}{rK(F_0 - F_e)}\ln\frac{C_0}{C} + \left[\frac{E}{r} - \frac{1}{rK(F_0 - F_e)}\right]\ln\frac{F_0}{F_0 - F_e + \frac{C}{F}}, \qquad \text{(VII)}$$

which shows the relation between the lowering of H_2O_2 concentration and the duration of the enzymatic process.

If $(F_0 - F_e) \to 0$, then equation (VII) at the end gives:

$$t = \frac{E}{r}\ln\frac{C_0}{C} + \frac{E}{rK}\left(\frac{1}{C} - \frac{1}{C_0}\right). \qquad \text{(VIII)}$$

If, however, $F_0 - F_e = 1/KE$, then equation (VII) converts into equation:

$$t = \frac{E}{r}\ln\frac{C_0}{C}. \qquad \text{(IX)}$$

When the enzyme is not inactivated during the reaction, i.e., when its catalytic capacity $E = \infty$, equation (IV) converts into the equation:

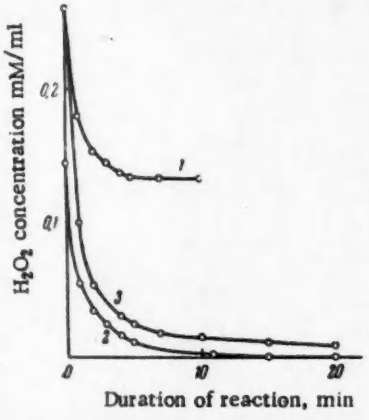

Fig. 1. Changes in H_2O_2 concentration during its decomposition by a catalase preparation at 37° and pH 6.8. Theoretical curves: 1) for $F_0 - F_e = -0.00108$; 2) for $F_0 - F_e = 0$; 3) for $F_0 - F_e = -0.00008$ ml preparation per ml of reaction mixture; circles - experimental data.

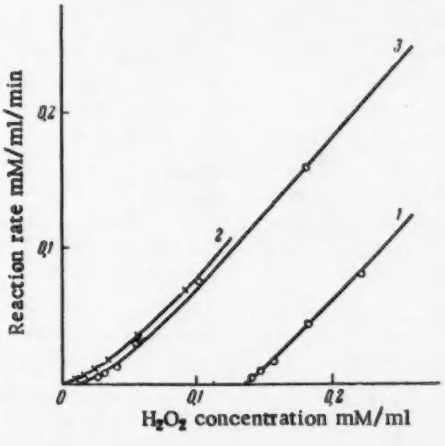

Fig. 2. Changes in the rate of H_2O_2 decomposition by a catalase preparation at 37° and pH 6.8. Theoretical curves same as in Fig. 1; crosses and circles - experimental data.

TABLE 2. Changes in H_2O_2 Concentration and in the Rate of Its Decomposition by Catalase at 37° and pH 6.8. Conditions of the enzymatic reaction: F_0 = 0.001 ml preparation per ml reaction mixture; E = 125 mM H_2O_2 per ml preparation; C_0 = = 0.26 mM per ml; $F_e = (0.26)/(125) = 0.00208$ ml preparation per ml reaction mixture; $F_0 - F_e = 0.001 - 0.00208 = -0.00108$ ml preparation per ml reaction mixture. Values of constants: K = 20; r = 150; $rK(F_0 - F_e) = -150 \times 20 \times 0.00108 = -3.24$. Formula (VII) for calculation of concentration: $t = \frac{2,3}{rK(F_0 - F_e)} \lg \frac{C_0}{C} + \left[\frac{2,3 \cdot F}{r} - \right.$ $\left. - \frac{2,3}{rK(F_0 - F_e)}\right] \cdot \lg \frac{F_0}{F_0 - F_e + \frac{C}{E}}$; $t = -0,71 \lg \frac{0,26}{C} + 2,63 \cdot \lg \frac{0,001}{\frac{C}{125} - 0,00108}$.

Formula (IV) for calculation of rate: $v = r\left(F_0 - F_e + \frac{C}{E}\right) \frac{KC}{1+KC} = 150\left(\frac{C}{125} - \right.$ $\left. - 0,00108\right) \frac{20C}{1+20C}$.

Experimental data			Calculated data				
				t, min		V, mM/ml/min	
t, min	C, mM/ml	V, mM/ml/min	C, mM/ml	by experimental curve	by formula	by experimental curve	by formula
0	0,260	—	0,260	0	0	0,130	0,1260
0,5	—	0,080	0,200	0,65	0,67	0,067	0,0624
1	0,180	0,043	0,150	2,30	2,25	0,014	0,0135
2	0,154	0,017	0,140	3,70	3,50	0,004	0,00442
3	0,145	0,008	0,136	5,50	5,23	0,001	0,00088
4	0,138	0,004	0,135	—	∞	0	0
5	0,136	—					
7	0,135	—					
10	0,135	—					

TABLE 3. Changes in H_2O_2 Concentration and in the Rate of Its Decomposition by Catalase at 37° and pH 6.8. Conditions of the enzymatic reaction: F_0= 0.001 ml preparation per ml reaction mixture; E = 125 mM H_2O_2 per ml preparation; C_0 = = 0.125 mM per ml; $F_e = (0.125)/(125) = 0.001$ ml preparation per ml reaction mixture; $F_0 - F_e = 0.001 - 0.001 = 0$. Values of constants: K = 20; r = 150. Formula (VIII) for calculation of concentration: $t = \frac{2,3 \cdot 125}{150} \lg \frac{0,125}{C} + \frac{125}{150 \cdot 20}\left(\frac{1}{C} - \right.$ $\left. - \frac{1}{0,125}\right)$, from this $t = 1,92 \lg \frac{0,125}{C} + 0,0427\left(\frac{1}{C} - 8\right)$.

Formula (V) for calculation of rate: $V = \frac{r}{E} \cdot \frac{KC^2}{1+KC} = \frac{150}{125} \cdot \frac{20C^2}{1+20C} = \frac{24C^2}{1+20C}$.

Experimental data			Calculated data				
				t, min		V, mM/ml/min	
t, min	C, mM/ml	V, mM/ml/min	C, mM/ml/min	by experimental curve	by formula	by experimental curve	by formula
0	0,125	—	0,125	0	0	0,110	0,107
0,5	—	0,068	0,100	0,3	0,28	0,082	0,080
1	0,057	0,036	0,050	1,3	1,28	0,030	0,030
2	0,0355	0,017	0,025	2,8	2,71	0,010	0,010
3	0,024	0,009	0,010	5,3	5,04	0,0025	0,0024
4	0,016	0,006	0,005	10,6	10,89	0,0010	0,0005
5	0,012	—	0,0025	—	20,00	—	0,00014
6	—	0,002	0,001	—	46,39	—	0,000023
7	0,008	—	0,000	—	∞	0	0
9	—	0,001					
11	0,004	—					
15	0,003	—					
20	0,002	—					

TABLE 4. Changes in Hydrogen Peroxide Concentration and in the Rate of Its Decomposition by Catalase at 37° and pH 6.8. Condition of the enzymatic reaction: $F_0 = 0.002$ ml preparation per ml reaction mixture; E = 125 mM H_2O_2 per ml preparation; $C_0 = 0.260$ mM per ml; $F_e = (0.260)/(125) = 0.00208$ ml preparation per ml reaction mixture. $F_0 - F_e = 0.002 - 0.00208 = -0.00008$ ml preparation per ml reaction mixture. Values of constants: K = 20; r = 150; $rK(F_0 - F_e) = -150 \times 20 \times 0.00008 = -0.24$. Formula (VII) for calculation of concentration: $t = -9{,}583 \lg \frac{0{,}260}{C} + 11{,}503 \lg \frac{0{,}002}{\frac{C}{125} - 0{,}00008}$. Formula (IV) for calculation of rate: $V = r\left(F_0 - F_e + \frac{C}{E}\right)\frac{KC}{1 - KC} = 150\left(\frac{C}{125} - 0{,}00008\right)\frac{20C}{1 + 20C}$.

Experimental data			Calculated data				
				t, min		V, mM/ml/min	
t, min	C, mM/ml	V, mM/ml/min	C, mM/ml	by experimental curve	by formula (VII)	by experimental curve	by formula (IX)
0	0,260	—	0,260	0	0	0,244	0,252
0,5	—	0,160	0,200	0,25	0,28	0,180	0,182
1	0,100	0,076	0,150	0,50	0,54	0,123	0,126
2	0,054	0,030	0,100	1,00	1,13	0,070	0,0720
3	0,040	0,012	0,050	2,30	2,29	0,025	0,0240
4	0,031	0,0075	0,025	5,00	4,30	0,006	0,0060
5	0,025	0,005	0,020	6,00	5,41	0,003	0,00343
7	0,018	0,0027	0,015	8,20	7,68	0,001	0,00138
10	0,017	0,001	0,012	11,20	11,32	—	0,00046
15	0,011	—	0,011	—	14,42	—	0,00022
20	0,010	—	0,010	—	∞	—	0

tion (II) converts into equation (I). In this case, the dependence of the initial and of the proceeding reaction rates on the substrate concentration is described by the Michaelis-Menten equation. A change in the substrate concentration during the reaction process will be expressed by the equation:

$$t = \frac{1}{rKF_0}\ln\frac{C_0}{C} + \frac{1}{rF_0}(C_0 - C). \qquad (X)$$

Experimental verification of the kinetic equations. For the verification of the derived equations it is necessary to have experimental curves for coordinates C-t (H_2O_2 concentration-duration of the enzymatic process) and V-C (rate of reaction - H_2O_2 concentration) for various relations of catalase and H_2O_2 concentrations. The points necessary for plotting of curves for the coordinates C-t were obtained as a result of analysis of samples taken from the reaction mixture during various intervals. The techniques for conducting such experiments were described before.

During taking of the sample with a pipette and its transfer into the flask with H_2SO_4 solution, an error inadvertently entered the determination of reaction time. This error was strongly expressed during the investigation of the change in H_2O_2 concentration in the beginning, during the first minutes of the reaction. For this reason, the course of the curve in the time interval from 0 to 5 min was ascertained in separate experiments, which were conducted in parallel with the principal experiment.

A test tube with 9 ml of H_2O_2 solution and a thin-walled ampoule with one milliliter of diluted catalase preparation were placed into a thermostat at 37°. After warming for 10 min the ampoule was broken and the contents of the test tube were mixed rapidly. After a definite time interval, ascertained with a stopwatch, 5 ml of 50% H_2SO_4 was added to the test tube in order to stop the enzymatic reaction. Undecomposed H_2O_2 was titrated with 0.1N $KMnO_4$.

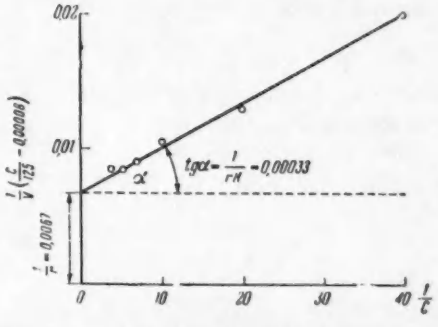

Fig. 3. Graphic determination of constants r and K. The straight line is drawn from experimental data in Table 4.

Experimental data concerning the changes in H_2O_2 concentration during the course of the enzymatic reaction are presented in Tables 2-4 and graphically in Fig. 1. According to the points on the experimental curve C-t, calculated the average reaction rate V_t at time t according to the formula:

$$V_t = \frac{C_{t-1} - C_{t+1}}{2},$$

where C_{t-1} and C_{t+1} are the H_2O_2 concentrations after t-1 and t+ 1 minutes, respectively. In the beginning of the enzymatic reaction, when decomposition of H_2O_2 proceeded at the fastest rate, the difference in concentration was taken at short time intervals (1 min), and at the end, when H_2O_2 decomposition was sharply reduced, this difference was taken at longer time intervals (4 min). The values obtained for the reaction rate are presented in Tables 2-4 and graphically in Fig. 2.

The constants r and K in the derived equations were obtained graphically. Experimental data were plotted in such coordinate systems that they would give straight lines. The coordinate system in each case was chosen from one or another kinetic equation. If $F_0 - F_e \neq \neq 0$, then for obtaining the constants r and K we chose coordinate systems stemming from equations (IV) and (VII). For example, equation (IV) is easily changed into the following equations:

$$\frac{1}{V}\left(F_0 - F_e + \frac{C}{E}\right) = \frac{1}{rKC} + \frac{1}{r}, \qquad \text{(XI)}$$

$$\frac{C}{V}\left(F_0 - F_e + \frac{C}{E}\right) = \frac{1}{rK} + \frac{C}{r}. \qquad \text{(XII)}$$

In accordance with equation (XI), experimental data concerning the dependence of V on C with given values for F_0, F_e, and E in the system of coordinates $\frac{1}{V}\left(F_0 - F_e + \frac{C}{E}\right) - \frac{1}{C}$ should give a straight line which, on the ordinate, will cut off a segment equal to 1/r and will have $tg\alpha = 1/rK$ (Fig. 3). In accordance with equation (XII), the same experimental data in the coordinates $\frac{C}{V}\left(F_0 - F_e + \frac{C}{E}\right) - C$ should give a straight line which, on the ordinate, will cut off a segment equal to 1/rK and will have $tg\ \alpha = 1/r$.

The values of the constants r and K can be found by the general method, starting with the Michaelis-Menten equation (I) and utilizing experimental data concerning the dependence of the initial reaction rate V_0 on the initial H_2O_2 concentration. The accurate determination of the constants r and K is quite a difficult problem. For their determination, experiments were repeated a number of times and the experimental data were corrected graphically. By plotting the data in the different coordinate systems, it was found that for the catalase preparation utilized in these experiments, the average values of the constants were: r = 125 and K = 20.

By substituting the found values of r and K into corresponding kinetic equations, we obtained the data (Tables 2-4) necessary for construction of the theoretical curves in the coordinates C-t and V-C (Figs. 1 and 2).

SUMMARY

The reaction of catalyzed decomposition of H_2O_2 was used to investigate the effect of reaction conditions on catalase inactivation. The study was conducted by determining the catalytic capacity (the maximum number of millimoles of H_2O_2 decomposed by 1 ml of a catalase preparation until its complete inactivation).

The catalytic capacity of catalase was found to be strongly reduced with an increase in temperature, with changes in the reaction medium towards the acid or alkaline regions, with the addition of enzyme poison (NaF, $HgCl_2$), but remained practically constant with changes in enzyme and H_2O_2 concentrations. The relation between the change of active catalase concentration and the change in H_2O_2 concentration during the course of the enzymatic process with given pH and temperature was thus found. A term accounting for the enzyme inactivation was introduced into the Michaelis-Menten equation.

Starting with the corrected Michaelis-Menten equation, we derived kinetic equations expressing the lowering of H_2O_2 concentration with increased duration of the enzymatic process. The derived kinetic equations were in good agreement with experimental data.

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POLAROGRAPHIC ANALYSIS OF PROTEIN SOLUTIONS WHICH HAVE BEEN SUBJECTED TO WEAK IONIZING RADIATION

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The indirect polarographic analysis of proteins which was proposed by Brdička [1] has been extensively developed. In a series of earlier studies [2, 3], we showed that with this method it is possible to establish definite properties of native proteins or to determine changes in protein structure which occur as a result of chemical or biological reactions. Taking into consideration the sensitivity of this method, we suggested that it could also be used to reveal the nature of the changes induced by weak ionizing radiation.

This is an account of results obtained in studies along this line.

METHODS

Proteins Studied and Their Preparation

Crystalline horse and bovine serum albumin. Albumin was obtained according to the method of Perez [4]. Before irradiation, solutions of 1% concentration were dialyzed in the cold against distilled water in the presence of xylol.

Crystalline β-lactoglobulin of cow's milk. The preparation was obtained by Palmer's method [5]. A 1% solution was dialyzed against 1% NaCl just before irradiation.

α-Globulin of horse and bovine serum. Preparation was obtained by precipitation with half-saturated $(NH_4)_2SO_4$ followed by dialysis against distilled water to a negative Nessler reaction.

α-Globulin of bovine sperm. Preparation was obtained as follows: 30-35 ml of freshly collected sperm was centrifuged and the supernatant was treated with half-saturated $(NH_4)_2SO_4$. The precipitate was redissolved and after filtration was once more precipitated with $(NH_4)_2SO_4$. The second precipitate was dissolved in a small quantity of distilled water and dialyzed against tap water to a negative Nessler reaction. The precipitate formed was removed by centrifugation, and the supernatant containing α-globulin collected. Homogeneity of the α-globulin was checked by paper electrophoresis.

Horse hemoglobin. Crystalline hemoglobin was obtained according to Drabkin [6]. The preparation was actually a mixture of hemoglobin and oxyhemoglobin. The crystals were dissolved and the solution subjected to dialysis against tap water.

Native casein of cow's milk. Milk freed of glycerides by centrifugation was diluted with water in the ratio 1:4. Casein was precipitated by acetic acid at pH 4.7. The precipitate was thoroughly washed several times with slightly acidified water, freed of lipids by washing with ethanol and ethyl ether, and dissolved in 1/15 M phosphate buffer, pH 6.8 .

Casein obtained after denaturation with chymosin was also used. It was extracted from Gruyere cheese, defatted, and dissolved in a 1% trisodium citrate solution.

Concentrations of protein solutions were determined by the Kjeldahl method. Irradiation was carried out with a Co^{60} source at a dose rate of 0.03 r/hour. The solution was irradiated in a polyethylene container. Time of exposure was accurately measured.

Polarographic determinations were made on a V-301 Heyrovski polarograph fitted with an adapter [7]. An alkaline solution prepared according to Brdička hexamino cobalt dissolved in ammonia buffer, pH 9.6, was used. Protein solutions analyzed polarographically were, as a rule, of known concentration.

In every case the capacity to suppress the cobalt maximum by half was determined, and also, if possible, the inversion point [8]. Other details of the polarographic measurements are presented in the figure legends.

RESULTS

To elucidate specific properties of the proteins studied and to facilitate comparison of the results obtained, a careful examination of the curves in the region between -1.3 volts and -1.9 volts, i.e., the region of polarographic catalytic activity of the proteins under consideration, was made. If the polarograms are obtained under the same conditions and drawn to the same scale, it is possible, by direct measurement of the height of the catalytic maxima and the potentials at which they appear, to compare the various proteins with respect to their structural peculiarities.

Horse serum albumin. Fairly soon after irradiation (5 minutes), the following changes were noted: decrease of the second maximum, increase in capacity to suppress the cobalt maximum, and decrease in the point of inversion. The changes are reversible, as shown by the tendency to return to normal, which is especially striking 24 hours after irradiation (Fig. 1).

Bovine serum albumin. Although the polarograms of horse and bovine serum albumin are different, the effects of irradiation on the second maximum and on C_S are similar in the two cases (Fig. 2).

β-Lactoglobulin of cow's milk. The polarogram of this protein is very specific (Fig. 3). In contrast to bovine albumin, there was a rapid increase in both maxima, and the capacity to suppress the cobalt maximum was retained. Return to normal was slow and the height of the maxima was clearly decreasing after 24 hours.

α-Globulin of horse serum. There was a decrease in the height of the first maximum and an increase in that of the second (Fig. 4). The capacity for suppression increased, and the inversion point rapidly decreased.

α-Globulin of bovine serum. As shown in Fig. 5, the height of the two maxima rapidly increases after a short period of irradiation and then rapidly returns to normal. The suppression capacity increases at first, and then it slowly decreases.

α-Globulin of bovine sperm. As shown in Fig. 6, the second maximum increases slightly, and with longer irradiation periods decreases. Both the suppression capacity and the inversion point rise rapidly.

Horse hemoglobin. As with the majority of proteins, the polarographic activity is significantly decreased as a result of irradiation (Fig. 7). The effect of irradiation is specific and is distinguished by the fact that the first maximum occurs at a more negative potential. This shift becomes more pronounced as time of irradiation is increased, indicating an important alteration in the macromolecular structure of the protein. As with other proteins, the suppression capacity is increased. After 24 hours the polarogram returns to normal; the suppression capacity is decreased.

Casein of cow's milk. This protein shows little polarographic activity (Fig. 8), and this is unaffected by irradiation; the height and position of the maxima show no change. At first the suppression capacity falls, and then it rises. In denatured casein from cheese, on the contrary, there is an increase in the height of the maxima and a rapid increase in the suppression capacity (Fig. 9). This demonstrates that the macromolecular structure of native casein is very stable and that only when the protein is denatured in a definite manner is it sensitive to radiation at the levels here employed. The causes of the exceptional stability of native casein are discussed elsewhere [3].

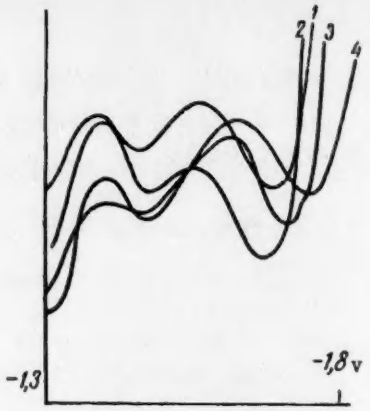

Fig. 1. Polarogram of crystalline horse serum albumin. Protein concentration – 6.45 mg%. 1) Before irradiation, C_S = 38.7 mg%, C_I = 3.22 mg%; 2) after a 5-minute irradiation, C_S = 25.6 mg%, C_I = 3.22 mg%; 3) after a 30-minute irradiation, C_S = 14.0 mg%, C_I = 3.22 mg%; 4) 24 hours after irradiation, C_S = =25.1 mg%, C_I = 3.22 mg%.

In this and the following figure legends, C_S denotes the capacity to suppress the cobalt maximum by half, expressed as protein concentration in mg% of an alkaline solution (Co^{+++}). C_I denotes the point of inversion of maxima, expressed as protein concentration in mg% of an alkaline solution.

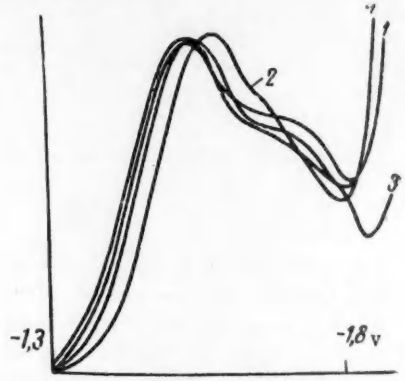

Fig. 2. Polarogram of crystalline bovine serum albumin. Protein concentration – 8.68 mg%. 1) Before irradition, C_S = 17.4 mg%; 2) after a 5-minute irradiation, C_S = 15.2 mg%; 3) after a 30-minute irradiation, C_S = 10.85 mg%; 4) 24 hours after irradiation, C_S = 13.05 mg%.

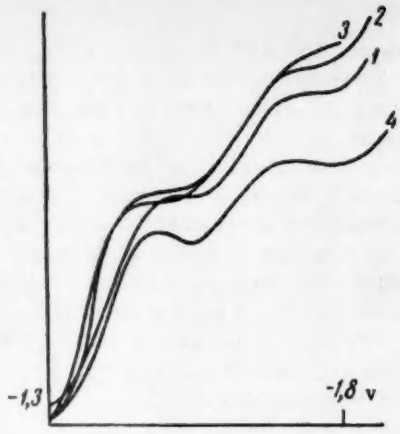

Fig. 3. Polarogram of α-lactoglobulin of cow's milk. Protein concentration — 6.20 mg %. 1) before irradiation, C_S = = 7.75 mg%, C_I = 1.55 mg%; 2) after a 5-minute irradiation, C_S = 8.50 mg%, C_I = 1.55 mg%; 3) after a 30-minute irradiation, C_S = 5.25 mg%, C_I = 1.55 mg%; 4) 24 hours after irradiation, C_S = = 6.50 mg%, C_I = 1.55 mg%.

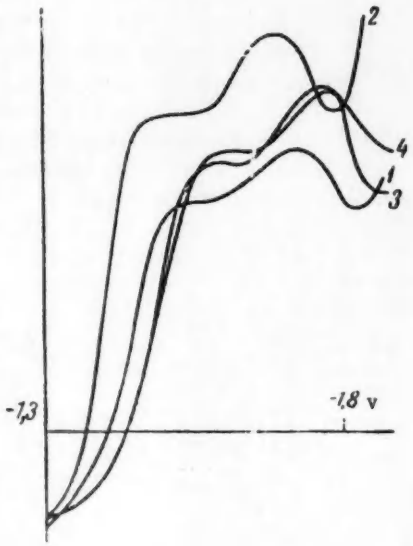

Fig. 5. Polarogram of α-globulin of bovine serum. Protein concentration — 5.15 mg%1) Before irradiation, C_S=15.45 mg% C_I=2.52 mg%; 2) after a 5-minute irradiation, C_S- = 13.35 mg%, C_I = 0.64 mg%; 3) after a 30-minute irradiation, C_S = 17.15 mg%, C_I = 1.30 mg%; 4) 24 hours after irradiation, C_S = 19.10 mg%, C_I = 2.52 mg%.

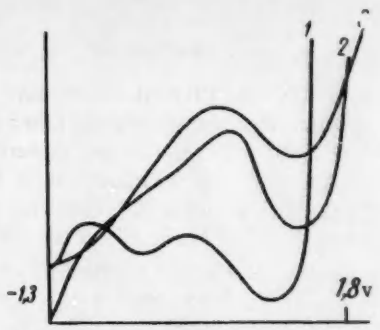

Fig. 4. Polarogram of α-globulin of horse serum. Protein concentration— —4.15 mg%. 1) Before irradiation, C_S = 28.6 mg%, C_I = 4.15 mg%; 2) after a 5-minute irradiation, C_S = 20.75 mg%, C_I = 1 mg%; 3) after a 30-minute irradiation, C_S = 16.6 mg%.

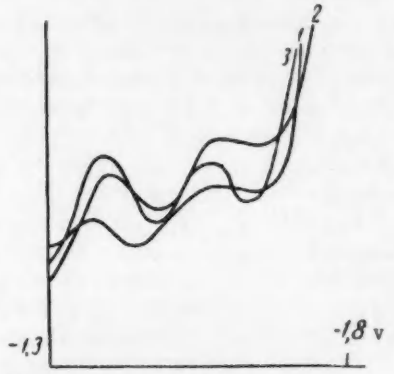

Fig. 6. Polarogram of α-globulin of bovine sperm. Protein concentration—4.72 mg%. 1) Before irradiation, C_S = 16.5 mg%, C_I = 2.36 mg%; 2) after a 5-minute irradiation, C_S = 5.90 mg%, C_I = 1.20 mg%; 3) after a 30-minute irradiation, C_S = = 16.25 mg%, C_I = 0.47 mg%.

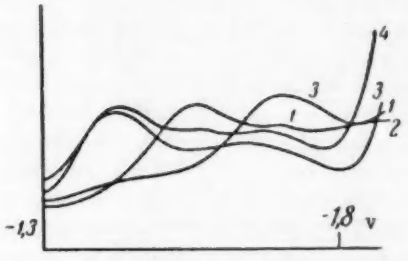

Fig. 7. Polarogram of horse hemoglobin. Protein concentration—6.55 mg%. 1) Before irradiation, C_S = 16.55 mg%; 2) after a 5-minute irradiation, C_S = 10.45 mg%; 3) after a 30-minute irradiation, C_S = = 13.0 mg%; 4) 24 hours after irradiation, C_S = 14.75 mg%.

DISCUSSION

Pavlovskaya and Pasynskii [9, 10] have shown that ionizing radiation has an associative effect on protein molecules. Since that time several workers have confirmed this, usually with radiation in large doses (exceeding 1000 r). We have shown this to be true with small radiation doses. Only in the case of one protein, native casein, was there a decrease in molecular weight with irradiation. This is due to peculiarities in the macrostructure of this protein [3].

The associative effect is reversible and can be repeated several times; this is an indication of the exceptional plasticity of the various proteins in their reaction to weak ionizing radiation and also of a high sensitivity to the latter.

With the polarographic method it is possible to make certain assumptions relative to the mechanism of this phenomenon. The majority of workers agree that the first maximum of the protein solutions is caused by various polyoses of glucido-protein complexes, although some workers [11] question this view. With respect to the second maximum, it is ascribed mainly to thiol groups, although Büchner et al. [12] have shown that uric acid and clupein, which do not contain sulfur, give polarographic curves similar to that of cysteine.

We believe that molecular structure, as well as chemical composition, plays a great role in the determination of the position and size of maxima. As established for purified proteins, the second maximum may be caused by thiol groups, with their position in the molecule exerting an effect on the polarographic parameters. As Rodionov and Kedrova demonstrated [13], the effect of radiation varies depending on whether the thiol groups are bound or free. This same phenomenon may be involved in the various effects of thiol coenzymes in inhibiting enzymes [14, 15].

Our results show that the effect of ionizing radiation on thiol groups is due not to their oxidation but to a change in proton mobility, which agrees with the recent studies of Romani and Tappel [16]. This non-specific denaturation could be explained by a widespread blocking leading to molecular association of the irradiated proteins. Normalization of the second maximum is accompanied by a decrease in the capacity to suppress the cobalt maximum (decrease in molecular weight). It should be emphasized that the first maximum also undergoes alterations; these indicate that the density and mobility of protons of the protonotropic groups of category I, which are responsible for the first maximum, are changed. The hypothesis that the primary effect of weak ionizing radiation is on macromolecular structure is thus confirmed. These changes vary with the nature of the protein and may be easily detected by polarography.

From the results it may be concluded that the effect of ionizing radiation on protein solutions varies with the protein. The rapid formation of aggregates of molecular weight exceeding that of the native protein has been observed. At low radiation intensities, this is a reversible process.

Macromolecular aggregation elicits steric modifications of the protein molecules which are accompanied by changes in proton mobility. These latter are involved in the catalytic effect of alkaline protein solutions. This explains the change in height and potential of polarographic maxima of the proteins studied.

The results obtained show that the polarographic method is adaptable to the study of the effect of radiation on protein solutions.

SUMMARY

Changes induced by weak ionizing radiation (Co^{60}) are specific for the proteins studied and are expressed in

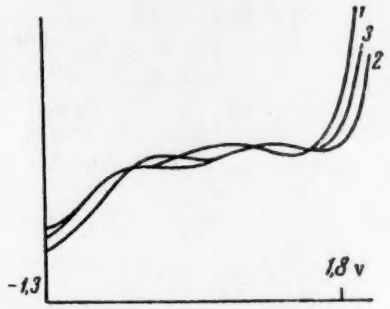

Fig. 8. Polarogram of casein of cow's milk. Protein concentration – 6.35 mg%. 1) Before irradiation, C_S = 15.87 mg%; 2) after a 45-minute irradiation, C_S = 19.20 mg%; 3) 24 hours after irradiation, C_S = 12.70 mg%.

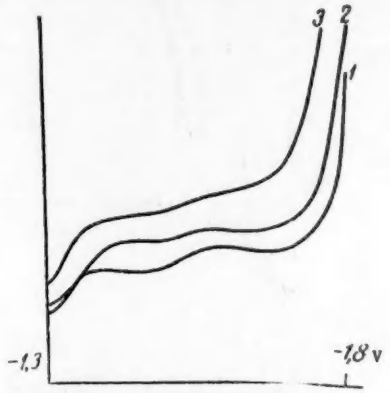

Fig. 9. Polarogram of casein of cheese from cow's milk. Protein concentration – 6.40 mg%. 1) Before irradiation, C_S = 14.40 mg%; 2) after a 45-minute irradiation, C_S = 12.80 mg%; 3) 24 hours after irradiation, C_S = 9.90 mg%.

the reversible formation of macromolecular aggregates. Polarographically this is expressed as an increase in the capacity to suppress the cobalt maximum and as a change in the height and potential of the catalytic maxima. These changes are due to changes in mobility of protons of protonotropic groups and to the formation of macromolecular aggregates.

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THE EFFECT OF FREEZE-DRYING ON THE SUCCINIC DEHYDROGENASE ACTIVITY OF Escherichia coli

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One of the most important points in the problem of the preparation of dry live vaccines consists in the preservation of the viability of the microbial cells during drying from a frozen state. A number of review articles [1] are devoted to the effect on the bacterial cell of cold and freeze-drying.

During freeze-drying, the bacterial cells are subject to a number of factors affecting their viability. Among these should be listed, to begin with, the effect of hypertonic solutions of electrolytes (during the formation of inter-and intracellular ice) on the proteins, lipoproteins, and colloidal structures; the effect of low temperature, especially of "temperature shock", on the proteins and the kinetics of metabolic processes, and, finally, dehydration. The said effects can lead to a partial or complete denaturation of protein colloids, including enzymes, with a lowering of the viability of the cell as a direct result.

It is well known that various enzymes are affected by low temperatures in a different way. Some of them change their activity quite considerably, while others remain almost unaffected.

Data are available in the literature [2] dealing with the high sensitivity to cold of the enzymes associated with the mitochondrial tissue fraction.

Succinic dehydrogenase is very sensitive to the effect of cooling and freeze-drying [3-5].

It is very essential that the succinic dehydrogenase performs not simply in a mixture of enzymes, but under condtions of strict "architectural structure" [6]. Thus, the disruption of the protein of the enzyme or of the colloidal state of the structure of the whole complex can lead to a disturbance of the oxidative processes and to a lowering of viability. The role of succinic dehydrogenase in the cellular life processes and its sensitivity to low temperatures indicate the desirability of its study in bacteria under conditions of deep cooling and drying from a frozen state, in order to elucidate the mechanism of the disruption in viability under these conditions. The objective of the present investigation is to find out the existence of differences in the performance of the succinic dehydrogenase reaction by Escherichia coli prior and subsequent to drying.

METHODS

The determination of succinic dehydrogenase activity was conducted by means of the recently suggested compound 2, 3, 5 - triphenyltetrazolium chloride [7, 8]. The tetrazolium is reduced to red triphenylformazane, which is insoluble in water.

The investigation was conducted on E. coli 894, which is typical as to aspects of culture and biochemical properties. The culture was incubated for 17.5 hours at 37°C in meat-peptone agar media. For drying, the same culture was washed off by means of a 10% solution of sucrose with 1% gelatin, and the suspension was poured into 0.5-ml ampules. The preliminary freezing was conducted at -40°C for a period of 18-20 hours. The culture was subjected to a 24-hour drying in a chamber apparatus; the condenser was cooled by a mixture of dry ice and alcohol. The material was kept at -20°C in ampules sealed under a vacuum of 100-200 mm Hg.

The microbial mass was rinsed six times with saline for the purpose of removing the exogenous and endogenous metabolic products. The microbial suspension was diluted to a count of 12 billion bacteria per ml by means of a 0.066 M phosphate buffer of pH 7.05-7.10. Subsequently, all the reagent solutions were prepared from the stock solutions by using the same buffer. The bacterial concentration in the incubated mix constituted 1.5 billions/ml, the concentration of 2, 3, 5 - triphenyltetrazolium chloride being 1 mg/ml (2.98 micromoles/ml).

The incubation mix consisted of 0.25 ml bacterial suspension, 0.25 ml of a 0.8% tetrazolium solution, with the other reagents according to circumstances, and 0.066 M phosphate buffer of pH 7.05-7.1 to the 2-ml mark. The incubation was conducted under a layer of sterile vaseline oil for 60 min at 37°C. Following the incubation, 1.0 ml of the mix was removed from under the vaseline by means of a drawn-out pipette, and this was added to

TABLE 1. Effect of Calcium Ions, Cystine and Arsenite on the Succinic Dehydrogenase Activity of E. coli

Experimental material	Rate constant				
	control	cystine 10^{-4} M	activation %	arsenite $5 \cdot 10^{-4}$M	inhibition %
Fresh culture	0.0026	0.0035	40	0.0014	46
Fresh culture + Ca ions at 10^{-5}M	0.0028	0.0034	21.4	0.0010	64
Dried culture	0.0029	–	–	–	–
Dried culture + Ca ions at 10^{-5}M	0.0035	0.0037	8.8	0.0019	46

Footnote. The degree of activation (or inhibition) was calculated from the ratio of the rate of reaction constants.

1.0 ml of glacial acetic acid. Then two portions of toluene were added (1 and 2 ml), and the extraction of formazane was conducted with energetic brief shaking. The toluene layer was clarified by means of centrifugation or settling for a period of from 30 minutes to 1 hour. The photometric determination was conducted in a 2.5-ml cuvette. The formazane calibration curve showed a sensitivity within a range of from 5 to 80 micrograms/ml. Where the toluene-formazane solution was more concentrated, it was diluted 2-3 times.

RESULTS

Where a suspension rinsed six times, without succinate, was being incubated, there was no formation of formazane (a slight pink coloration after 24 hrs). Upon the addition of succinate, an intensive coloration of the mix began, reaching a maximum in 24 hours (an intensive red-violet coloration). No crystals of formazane could be detected microscopically outside of the cells.

The optimum hydrogen-ion concentration was checked by means of a 0.066 M phosphate buffer, the pH of which fluctuated from 6.0 to 9.0. The concentration of succinate in the incubation mix was 0.01 M. The results of the experiments are represented in Fig. 1, where it can be seen that the maximum formazane formation takes place at pH 7.1.

The effect of calcium ion, cystine and sodium arsenite on the activity of the enzyme. It is considered that calcium ions indirectly activate the enzyme by destroying Coenzyme 1 and by preventing the formation of oxalacetate, thus depressing the enzymatic activity. In our experiments, the calcium ion enters the incubation mix as $CaCl_2$ (by adding 0.1 ml of a 0.002 M solution of $CaCl_2$). The concentration of calcium ions in the mix was 10^{-5} M. In a concentration of 10^{-4} M, the calcium ions either did not affect the reduction of tetrazolium, or depressed it slightly. The effect of cystine was studied in the presence and in the absence of calcium ions in the medium.

Arsenite is a well-known inhibitor of succinic dehydrogenase. We studied the effect of various concentrations of sodium arsenite on the reduction of tetrazolium in the presence and absence of calcium ions in the medium. Preliminary tests revealed that in 10^{-3} M concentration, the arsenite depressed the reaction by 80-100%, and at 10^{-4} M, by 5-20%. We selected the mean concentration of $5 \cdot 10^{-4}$ M. The period of contact with the arsenite previous to incubation amounted to 30 minutes. The results of the experiments are summarized in Table 1.

It can be seen from the reported data that in a dried preparation, the rate of reaction increases upon the addition of calcium ions to a much larger degree than in a fresh preparation. Cystine exerts an activating influence on the succinic dehydrogenase activity, and this effect is much more pronounced in a fresh culture than in a dried one. In the absence of calcium ions in the media, the activation is much more pronounced than in their

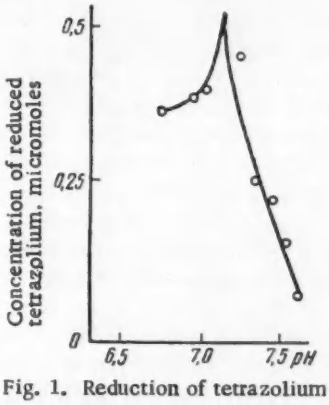

Fig. 1. Reduction of tetrazolium by a suspension of E. coli 894 at various pH values. Concentration of succinate in the medium is 0.01 M. Incubation at 37° for a period of 60 min. Each point is the average of 3-5 experiments.

presence (in a fresh culture). The literature [9] mentions the inhibitory effect of cystine on the tissue succinic dehydrogenase. Sodium arsenite depresses the enzyme considerably, and this depression is somewhat more pronounced in a fresh culture than in a dried one. In a fresh culture containing calcium ions, the depression of the enzyme is much more pronounced than in the absence of calcium ions.

In connection with the revealed differences in the activity of the enzyme, it was desirable to study a few points of the kinetics of the reduction reaction of tetrazolium in fresh and dried cultures. For this purpose we studied the effect of the concentration of the substrate (sodium succinate) on the rate of tetrazolium reduction. The results of our experiments are represented in Figure 2, from which it can be seen that there is a considerable difference in the rate of tetrazolium reduction by dried and fresh preparations.

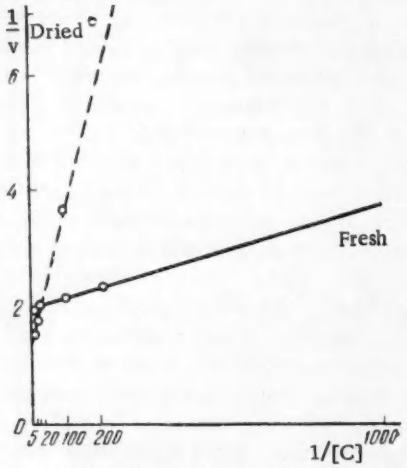

Fig. 2. The relation of the rate of the tetrazolium reduction reaction to the concentration of succinate. Dotted line) reduction of tetrazolium by a dried cultyre of E. coli 894; solid line) reduction by a fresh culture. The curves are constructed according to the method of Lineweaver and Burk. Abscissae) Values inverse to the concentration of the substrate (moles); ordinates) values inverse to the reaction rate (expressed as micromoles of reduced tetrazolium). Each point is the average of eight experiments.

The method of Lineweaver and Burk [10] was used for the calculation of the dissociation constant of the enzyme-substrate complex, applying the expression:

$$\frac{1}{v} = \frac{K_M}{V}\frac{1}{[C]} + \frac{1}{V}$$

where v is the reaction rate of the reduced tetrazolium, expressed in micromoles; V is the maximum reaction rate, [C] is the concentration of the substrate, and K_M is Michaelis' constant.

TABLE 2. The Rate of Tetrazolium Concentration Related to the Concentration of Sodium Succinate.

Conc. of succinate (M)	Conc. of reduced tetrazolium (μM/ml)	
	fresh culture	dried culture
0,001	0,296	0,139
0,005	0,427	0,137
0,01	0,460	0,271
0,05	0,500	0,578
0,1	0,510	0,612
0,2	0,430	0,707
0,3	—	0,646

The experimental data of Table 2, substituted into the expression, made it possible to construct the corresponding graph (Fig. 2). It is possible that the reaction is not of the first order, but the experimental data (Table 2) fit well into the selected equation.

Using the method of least squares, we calculated by means of the said expression the value of Michaelis' constant for both preparations, obtaining, for the fresh culture, $K_M = 6.5 \cdot 10^{-4}$, and, for the dried culture, $K_M = 2.4 \cdot 10^{-2}$.

SUMMARY

2, 3, 5-Triphenyltetrazolium chloride is a specific reagent for assaying the succinic dehydrogenase activity of E. coli. No formazane is formed in the absence of succinate. By means of this compound the optimum pH of the enzyme was established and the effect of certain compounds was investigated. Ca^{++} and cystine greatly enhance the reduction of tetrazolium. Na arsenite inhibits the reaction. Certain differences have been found in the activation and inhibition of the reaction in fresh and freeze-dried cultures of E. coli. The dissociation constant of the enzyme-substrate complex is much higher in the freeze-dried culture than in the fresh one.

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THIOL SYSTEMS OF ERYTHROCYTES AND THE FORMATION OF METHEMOGLOBIN

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The formation of methemoglobin (MtHb) is assumed to be a process of reaction of oxidizing agents with a prosthetic group of hemoglobin.

However, at the present time there is reason to believe that this is an effect of considerably greater complexity. In this connection, especially, it is interesting to discover what part the thiol systems of erythrocytes play in the formation and transformations of methemoglobin.

One of the representatives of such systems is SH-glutathione, which was discovered in 1925 by Holden [1] in blood erythrocytes. In 1938 Morrison and Williams [2] established the ability of glutathione to reduce methemoglobin to hemoglobin and expressed the idea that glutathione participates in the mechanism which prevents hemoglobin from oxidation.

To the number of thiol systems of erythrocytes can also be added the group of dehydrogenases, whose share in the reduction of methemoglobin has been definitely indicated [3]. A number of works have been devoted to this in recent years. It has been established that the protein portion of hemoglobin contains SH groups, both "freely reactive" [4, 5] and "masked" [6, 7]. Eggleton and Fegler [8] suggested that the activity of hemoglobin could be connected to a considereable extent with the SH groups of its protein portion, which, in their opinion, would permit an understanding of the protective role of SH-glutathione in the formation of methemoglobin. The hypothesis of these authors was confirmed by the report that the binding of oxygen by dry hemoglobin can be markedly lowered by preliminary blocking of the SH groups of globin by thiol poisons [9]. Meyer-Wilmes [10] considers as likely the possibility of oxidation of SH groups of hemoglobin by methemoglobin-forming substances.

In connection with these reports, the problem of our investigation is the determination of the content of SH groups in erythrocytes in methemoglobin formation.

METHODS

Content of SH groups in erythrocytes in methemoglobin formation. The experiments were carried out on washed dog erythrocytes. As the methemoglobin-forming agent we used sodium nitrite. Using a Pulferich spectrophotometer with a 619 mμ light filter, we carried out a determination of the methemoglobin content under the action of nitrite, taken in the amounts of 2, 4, 8, and 12 mole/mole hemoglobin. In parallel with these samples we determined the content of SH groups by the method of amperometric titration with a 10^{-3} M solution of silver nitrate according to Kolthoff and Harris [11]. For this the erythrocytes were hemolyzed with 20 times the volume (of the suspension) of distilled water, the hemolyzate was centrifuged at 6000 rpm, and 0.5 ml of the upper layer of liquid was drawn off for titration. The results of the series of experiments are given in Fig. 1.

Content of SH groups in the erythrocytes in reduction of the methemoglobin. Having observed the oxidation of SH groups of erythrocytes by the methemoglobin forming substance, we used sodium sulfite for their reduction, as used by Kolthoff and Stricks [12] for the reduction of disulfide groups to SH groups.

The experiments were carried out as described above, after washing out the excess methemoglobin-forming substance and bringing the volume of the suspension up to the original; the erythrocytes were kept for ten minutes in the presence of sulfite taken as calculated for 2 moles/mole hemoglobin. Then the erythrocytes were washed and the content of SH groups and methemoglobin were determined. Fig. 2 gives the resulting data.

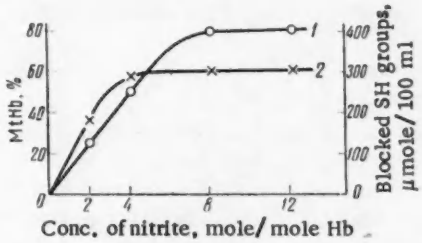

Fig. 1. Content of SH groups in erythrocytes in methemoglobin formation. Each point represents an average value from the results of 7 experiments. 1) MtHb content; 2) content of blocked SH groups.

Comparative Rate of Reduction of Methemoglobin and Blocked SH Groups of Erythrocytes by Some Reducing Agents. Values given are averages of seven experiments.

Reducing agent	Amount of MtHb, %		Amt. of SH groups, %		Reduction of MtHb, %	Reduction of SH groups, %
	before reduction	after reduction	before reduction	after reduction		
Ascorbic acid	32,2 ± 2,5	30,6 ± 1,8	79,8 ± 2,1	81,1 ± 1,5	1,6	2,1
Glucose	32,2 ± 2,5	31,5 ± 1,5	79,8 ± 2,1	82,9 ± 1,3	0,7	3,1
Na_2SO_3	32,2 ± 2,5	22,6 ± 2,4	79,8 ± 2,1	94,0 ± 1,5	9,6	14,2

Formation of methemoglobin after preliminary partial inactivation of the SH groups of the erythrocytes. For a study of the effect of preliminary inactivation of the sulfhydryl groups of the erythrocytes on methemoglobin formation, we ran a series of experiments using thiol poisons with an alkylating (iodoacetic acid) and mercaptide-forming (mercuric chloride) type of action. The substance, taken at 1 mole per 4 moles hemoglobin, was dissolved in a suspension of erythrocytes, and the suspension was then kept at room temperature for a day. Then the erythrocytes were washed and the suspension, whose volume was brought to the original, was submitted to the action of nitrite. The results are shown in Fig. 3.

Comparison of the effectiveness of some reducing agents for methemoglobin. In the last series of experiments, for determination of the rate of reduction of methemoglobin, we used preparations of ascorbic acid, glucose, and also sodium sulfite.

After formation of the methemoglobin (2 moles $NaNO_2$/mole hemoglobin) and washing of the cells, the erythrocytes were kept 10 min in the presence of an equimolar amount of the reducing agent (1.5 mole/mole hemoglobin). After washing the erythrocytes we determined the content of methemoglobin and SH groups (Table).

RESULTS AND DISCUSSION

As Fig. 1 shows, the action of increasing concentrations of methemoglobin-forming substances is accompanied in our experiments by progressive inactivation of the SH groups of the erythrocytes. This fact indicates the undoubted participation of the thiol system of erythrocytes in the reaction with methemoglobin-forming substances. At the same time, the parallelism between formation of methemoglobin and blocking of SH groups shows the close interrelation of these two processes. This interrelation is retained also during the reverse reaction: Reduction of methemoglobin and increased number of titratable SH groups are also parallel processes (Fig. 2).

If these findings confirm the correctness of the hypothesis of the connection between the thiol systems of erythrocytes and the process of oxygenation of blood, then the results of the experiments with the formation of methemoglobin based on preliminary blocking of the erythrocyte SH groups, as it seems to us, can be interpreted to indicate the occurrence of a share by the thiol system of the erythrocytes in the formation of methemoglobin. From the course of the curves given in Fig. 3 it is evident that the preliminary partial inactivation of erythrocyte SH groups by thiol poisons leads first to spontaneous formation of methemoglobin, and second, to a sharp increase in its content under the action of small concentrations of methemoglobin-forming compounds (on the average, double).

In the light of these data, the question arises as to whether there is not a sort of "thiol effect" in blood

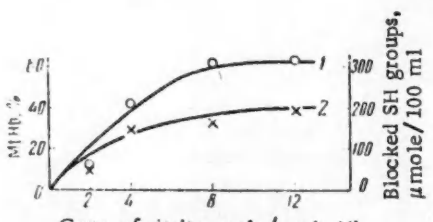

Fig. 2. Content of SH groups in erythrocytes in reduction of methemoglobin. Symbols the same as in Fig. 1.

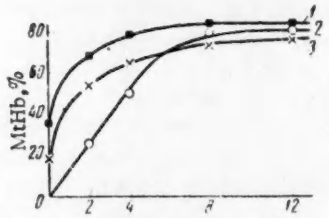

Fig. 3. Formation of methemoglobin after preliminary partial inactivation of SH groups of erythrocytes by mercuric chloride (1) and iodoacetic acid (3). For comparison we give the curve of formation of nitrite MtHb in control experiment (2).

oxygenation. Here, as in a whole series of biological reactions which depend on an active SH group, there is a characteristic regularity: Inactivation of the erythrocyte thiol system under the influence of agents which oxidize SH groups destroys the process of oxygenation due to the formation of methemoglobin, and their reactivation is accompanied by a return to normal of this process due to reduction of the methemoglobin.

For an understanding of the details of the mechanism of this effect, further studies are necessary, However, in the light of the reports of a number of authors, the following can be established at once. First, hemoglobin in erythrocytes is protected from oxidation by thiol systems, by fixed stroma [8]. In just this way Morrison and Williams [2] explained the spontaneous formation of methemoglobin (from oxidation by oxygen of the air) in the hemolysis of erythrocytes. Hence, oxidation of hemoglobin can in considerable degree be facilitated by blocking the "protective" SH systems with thiol poisons. Second, we see the role which the SH systems of the protein portion of hemoglobin play in methemoglobin formation.

It is known that the ability of hemoglobin to bind molecular oxygen reversibly without change in the valence of the iron of the heme depends on the specific structure of the globin. Denaturation of the globin leads to loss of this ability and oxidation of the heme [13]. On the other hand, a number of authors have considered the significance of the SH groups of proteins for their aggregate state. Thus, in the opinion of Rodionov and Kedrova [14] inactivation of the SH groups of a protein is one of the reasons for the destruction of intermolecular bonds of the protein molecule and disorganization of its biological structure. As Simpson and Saroff showed [15], inactivation of the SH groups of proteins is the first step in their denaturation. This is also indicated by the data of Kolthoff, Anastasi, and Tan [16]. There is also a direct relation to this in the observation of Shapot [17] of the easier denaturation of methemoglobin compared to hemoglobin.

In the titration of SH groups in our experiments we have evidently dealt especially with the SH groups of hemoglobin, since the "protective" SH systems of stroma were removed in centrifuging the hemolyzate. However, for a final solution of the question of the role of SH groups of globin in the reaction of methemoglobin formation we must have experiments with preparations of dry, purified hemoglobin.

Finally, we should observe that the intensity of reduction of methemoglobin, as the data of the table show, is connected to a considerable extent with the ability of the reducing agent to reactivate the oxidized SH group. The action of such specific reducers of the disulfide grouping as sodium sulfite, as we have seen, produces reduction of methemoglobin even at very short exposures, when the reducing effect of glucose or ascorbic acid has not yet appeared. However, in this connection we should consider the opinion of Mills and Randall [18] according to which the action of glucose leads to retaining glutathione in the erythrocyte in the reduced form, that is, is also connected with an effect on the thiol system of the erythrocytes.

SUMMARY

The formation of methemoglobin is accompanied by inactivation of the sulfhydryl groups of the erythrocyte thiol systems, and the reduction of methemoglobin, by their reactivation.

Blocking the erythrocyte sulfhydryl groups by thiol poisons of the alkylating and mercaptide-forming types considerably increases the formation of methemoglobin when followed by the use of methemoglobin-forming substances.

The thiol systems of erythrocytes play an important role in the reaction of formation and reduction of methemoglobin.

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THE CONVERSION OF INOSINATE TO ADENYLATE IN MUSCLE EXTRACTS*

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Intensive functional activity or some types of damage of muscle and of other tissues are accompanied by a conversion of ATP to AMP, followed by the enzymatic deamination of the latter. A number of authors [1-3] consider the deamination of AMP as one of the physiologically important sources of body ammonia.

The reverse of the above reaction, namely, the resynthesis of AMP from IMP, is of no lesser importance, since it serves the maintenance of normal tissue functions. Yet, although the enzymatic breakdown of AMP has been studied in great detail, no accurate knowledge of the reverse process was available until recently.

Of great assistance in the elucidation of this reaction has been the application of chromatographic and tracer techniques for the study of purine nucleotide biosynthesis. It was shown in recent years that both the neosynthesis of AMP and its resynthesis in tissues occurred via IMP amination. The conversion was shown [4] to take place through the condensation of IMP with L-aspartate to form an intermediate, adenylosuccinate, the latter being cleaved enzymatically to AMP and fumarate: A: IMP + L-aspartate + ATP (GTP) $\rightarrow P_i$ + ADP (GDP) + adenylosuccinate. B: adenylosuccinate $\rightleftharpoons$ AMP + + fumarate.

The amination of IMP was shown to be coupled with the dephosphorylation of ATP in bone marrow [5] and in muscle [6], while in bacteria, GTP participates [7] (equation A above). In the reports cited [5-7] the actual net synthesis observed was low and the conversions could only be traced by the use of isotopes (C^{14}-IMP or N^{15}-aspartate).

In view of published evidence [8, 9] about the participation of amino-nitrogen sources other than aspartate in AMP resynthesis from IMP in muscle, the present work dealt with the optimal conditions of this reaction, with the specific participation of aspartate in it and with the effect on the reaction of certain aspartate antimetabolites, namely α-me-DL-asp β-me-DL-asp and L-cysteic acid. The ultimate aim of the present work was to establish the possible importance of indirect deamination via AMP [10] in the general dissimilation of amino acid nitrogen in animals.

EXPERIMENTAL

Enzyme Preparations

Muscle extracts. IMP amination was studied with an enzymatic preparation obtained by acetone treatment of pigeon breast muscle extracts [6]. Pigeon breast muscle was homogenized three minutes in three volumes of 3×10^{-3} M EDTA. The homogenate was centrifuged 15 min at 11,000 rpm ; the addition of six volumes of cold acetone to the supernate produced a precipitate which was dried in air and stored in a desiccator in the cold. On the day of the experiment, 0.4 g of the acetone powder was extracted with 2 ml of water in a mortar and centrifuged for five min at 6,000 rpm . The supernate was dialyzed 1.5 h against cold distilled water according to the rapid method of Gorkin [11]. The dialyzed extract (AE) was used immediately.

Potato apyrase. Apyrase was prepared according to Lee and Eiler [12]. Aqueous solutions of this enzyme were concentrated five to seven fold by evaporation in cellophane bags in a current of air at room temperature. The concentrated solution was stored in the cold under toluene. The activity of the enzyme preparation was assayed as its ability to split phosphorus from ATP under defined conditions.

Adenosine-6-phosphate deaminase (Adenylic deaminase). Solutions of reprecipitated rabbit muscle myosin [13] was used as a source of deaminase without it the removal of the ATP ase present. The myosin solutions were divided into separate portions and kept frozen. The activity of the enzyme was assayed by incubation with a standard AMP solution and determination of ammonia formed under defined conditions.

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* The following abbreviations will be used: ATP) adenosine triphosphate; AMP) adenosine monophosphate; IMP) 5-inosinic acid; GTP-guanosine triphosphate; PGA-3-phosphoglyceric acid; apyrase) adenylpyrophosphatase; AE) muscle acetone powder extract; EDTA) disodium salt of ethylenediamine tetraacetic acid; α-Me-DL-Asp and β-Me-DL-Asp) the α-and β-methyl-DL-aspartate, respectively.

METHODS

Incubation mixtures (total volume 1.5 ml) contained 0.2 ml of AE, 10 μ moles of Na-PGA, 7.5-10 μ moles of IMP, 3-10 μ moles ATP, $MgCl_2$ and L-aspartate, 2×10^{-2} M phosphate buffer, pH 7.4. Mixtures were incubated for periods ranging from 1 to 4 hours at room temperature (22-24°). The reaction was stopped by immersion in boiling water for one minute, and the nucleotide composition of the reaction mixtures was determined enzymatically (Method) or chromatographically (Method II).

Method I. The mixtures were adjusted to pH 6.5 with 0.5 N HCl; To each mixture were then added 0.5 ml of 0.2 M succinate buffer (pH 6.5), 0.1 ml of 0.5% $CaCl_2$, apyrase solution (0.5 to 1.0 ml, depending on the activity of the preparation) and 0.1 to 0.2 ml of the myosin solution. The mixtures were incubated 60 min at 37° and the reaction stopped by the addition of TCA. Portions of the TCA filtrates from experimental and control incubation mixtures were taken for spectrophotometric determinations and for ammonia determinations by the isometric distillation procedure of Conway followed by Nesslerization; the amount of AMP deaminated by myosin was calculated as μ moles per incubation mixture.

Method II. The initial stage of the experiment was as above, apyrase alone being used in the assay stage; the addition of myosin solution (adenylic deaminase) was omitted. The TCA filtrates were chromatographed on paper over periods of 72-96 hours in the solvent n-propanol – concentrated ammonia – 2×10^{-3} M EDTA (6:3:1) [14]. Leningrad "B" paper No. 2, washed with 0.5% EDTA (pH 8.5) and dried before use [15] was used for chromatography. The solutions (0.08–0.1 ml) were applied to the paper by micropipette in several applications with intermediate drying. The nucleotide spots were detected by their quenching in the ultraviolet when illuminated by a Brumberg lamp. The portions of chromatograms which contained AMP were excised, eluted with three ml of 0.01 N HCl and the AMP in the solutions was determined by its UV absorption at 260 mμ using a Beckmann-type photometer (SF-4). The ATP used in all experiments was a commercial preparation; IMP (Ba salt) was prepared from rabbit muscle according to Embden [16]; GTP was a gift from Prof. J.M. Buchanan, of the Massachussets Institute of Technology, U.S.A.

The barium salt of PGA (commercial preparation) was converted to the sodium salt prior to use. L-aspartic acid (from L-asparagine), L-cysteic acid (from L-cysteine), γ-aminobutyric acid (from L-glutamate, by enzymatic decarboxylation) and α-me-DL-asp [17] were prepared in the laboratory and purified by recrystallization. β-me DL-asp and D-aspartate were gifts from G.A. Galegov and from M. Lerman, respectively.

RESULTS AND DISCUSSION

Preliminary control experiments showed that when the reaction mixture contained energy-rich phosphorus sources such as ATP and PGA, the AMP formed during incubation did not accumulate, but was rapidly phosphorylated to ATP. The commercial ATP preparation used contained chromatographically demonstrable impurities of ADP and AMP. After the incubation of such preparations with the AE preparation in the presence of PGA, only one spot, corresponding to ATP, could be identified chromatographically. Thus the entire complement of adenine nucleotides initially present in the reaction mixture – whether introduced with the ATP supplied, or endogenous material present – was finally converted into one sole form, namely ATP. The reaction mixtures investigated were capable of such rapid phosphorylation of AMP in the presence of PGA that all the AMP present (added with the ATP or produced during incubation) was immediately removed and no ammonia formation in mixtures incubated with AE could be detected despite the fact that an active deaminase was present in the AE preparation. These results also served as controls in that they indicated that neither the L-aspartate added, nor any other preformed ammonia source was capable of releasing ammonia under the experimental conditions.

The present experiments confirmed the findings of other authors about the lack of resolution of ATP from IMP when these compounds are chromatographed on paper in the solvent of Hanes and Isherwood [14]. It was therefore not possible, under the present conditions, to determine the increase in ATP at the expense of IMP by means of simple chromatography followed by spectrophotometric assay of nucleotide in the eluate from the respective areas.

In view of this difficulty, the total increase in adenine nucleotide (which reflected the quantitative conversion of IMP to AMP) was assayed as the increase in total AMP released by potato apyrase after dephosphorylation of ATP to AMP. Reaction mixtures, previously incubated with AE, were boiled to inactivate the enzymes present, and then reincubated with excess apyrase for one hour. This procedure resulted in the quantitative conversion of the ATP present to AMP, which could be easily separated by paper chromatography [14] from other nucleotides, after which it was assayed spectrophotometrically (method II). The increase in AMP thus assayed was then compared with control incubations from which the IMP and L-aspartate were omitted.

Method I (above) was found to be more rapid and more suitable for multiple determinations. In this procedure all the adenine nucleotide present in the incubation mixture was reduced to the AMP state by incubation with apyrase. The resultant AMP was then quantitatively deaminated with adenylic deaminase and the determination of ammonia produced allowed calculation of the amount of AMP initially present and therefore the net conversion of IMP into AMP in the first incubation stage. Here again control incubations were carried out with reaction mixtures from which IMP and L-aspartate were omitted. The amount of apyrase and myosin solutions required for complete assay was determined in

TABLE 1. AMP Synthesis from IMP and L-Aspartate in Pigeon Muscle Extracts. (Reaction mixtures contained: ATP, 5 μmoles; IMP, 10 μmoles; L-aspartate, 5 μmoles. Incubation time—one hour). Adenine nucleotides assayed by Method I

Expt. No.	ΔNH_3 (ΔAMP) μmoles	Expt. No.	ΔNH_3 (ΔAMP) μmoles	Expt. No.	ΔNH_3 (ΔAMP) μmoles	Expt. No.	ΔNH_3 (ΔAMP) μmoles
1	1,50	6	0,72	11	0,73	16	1,66
2	2,10	7*	1,11	12	0,71	17	1,27
3	0,74	8	1,53	13	1,86	18	1,41
4	0,87	9*	1,31	14	1,11	19	1,63
5	0,63	10	1,70	15	0,76	20	1,36

*L-aspartate, 3 μmoles; IMP, 7.5 μmoles.

preliminary test incubations involving the additions of known amounts of ATP and IMP to heat-fixed incubation mixtures.

A number of experiments were carried out in order to determine substrate concentrations and other experimental conditions (listed in "Methods" above) necessary for optimal AMP synthesis from IMP and L-aspartate. The reaction was shown to be enzyme-dependent since boiling of the extract abolished all activity. Ammonia formation could not be demonstrated (using Method I) if L-aspartate was omitted. A slight amount of ammonia (i.e., AMP) was assayed in incubation mixtures from which IMP was omitted, but where L-aspartate was present. This was apparently due to the presence of some endogenous IMP in the AE preparation, which was confirmed by chromatography of its TCA extract.

The effect of varying concentrations of L-aspartate on the synthesis of AMP was also investigated. In the presence of three μmoles of ATP and seven μmoles of IMP, the reaction mixture became saturated with L-aspartate when the concentration of the latter reached 2×10^{-3} M, (three μmoles in 1.5 ml); further increase in L-asparate concentration caused only a very slight increase in the amount of AMP synthesized in either one or three hours. When the IMP concentration was decreased to five μmoles per test, AMP synthesis fell, as a rule. Maximal AMP synthesis occurred in the presence of 7.5—10 μmoles of IMP.

In incubation mixtures containing three μmoles of L-aspartate and incubated three hours, AMP synthesis remained almost unaffected by alteration of pH between 6.9 and 8.6.

The values for AMP synthesis from IMP obtained in a number of reaction mixtures containing dialyzed AE prepared from pigeon breast muscle and substrates in concentrations shown, and incubated for one hour, are given in Table 1. It will be seen from the table that individual AE preparations, although prepared according to a standard procedure, showed considerable scatter in activity. The activity of the dry powder remained generally constant during cold storage for at least several weeks, but in some cases a rather sharp fall in activity occurred.

The mean increase in AMP in the 20 experiments quoted was about 1.25 μmoles per incubation. This corresponded to the conversion of 25% (and in some experiments, 35—40%) of the L-aspartic acid added. As mentioned above, the extent of AMP synthesis in those experiments was measured indirectly as ammonia produced after incubation with deaminase.

Presented in Table 2 are the values for AMP synthesis obtained by each of the two assay methods used, namely, with apyrase and adenylic deaminase (Method I) and by the spectrophotometric method following the dephosphorylation with apyrase and paper chromatography (Method II). The values for AMP synthesis obtained by the two methods were found to be quite close, the divergence not exceeding 7%.

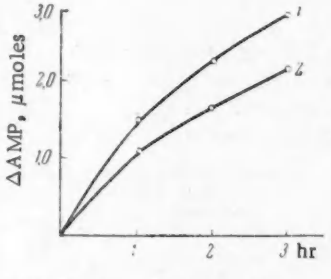
The progress of the reaction with time was also investigated. The amount of adenylic nucleotide formed increased steadily over the first three to four hours of incubation. In incubation mixtures containing three μmoles of L-aspartate, this incubation period brought about almost complete utilization of aspartate amino-nitrogen for IMP amination. Time-progress curves two similar experiments are shown in the figure.

As mentioned in the introduction, IMP amination to AMP in muscle extracts [6] and in bone marrow [5] is coupled with ATP breakdown, while in extracts of E. coli this process was shown to require GTP, which could not be replaced by ATP [7]. In the experiments described above, AMP formation in pigeon breast muscle extracts took place in the presence of catalytic amounts (1—3 μmoles per test) of ATP, its continuous regeneration being insured at the expense of phosphate transfer from PGA present to the products of ATP breakdown (or to the AMP generated de novo from IMP).

It was of interest to determine whether ATP in the present system could be replaced by GTP. In view of the small amounts of GTP available to us, only limited control experiments could be carried out with this compound. The results of three experiments involving GTP are presented in Table 3. In experiments 1 and 2 the incubation time was one hour, and the concentration of GTP employed was only one μmole per test (compared with 3.5 and 4.5 μmoles of ATP employed).

It will be seen from the results of Experiments 1 and 2 (Table 3) that, in the presence of ATP, added GTP somewhat inhibited IMP amination. In the absence of ATP the addition of one μmole of GTP caused some AMP synthesis, but that was considerably lower than that synthesized when ATP was present. In Expt. 3 the conditions were reversed: The ATP content was decreased to 2.5 μmoles, while the amount of GTP added was 3 μmoles; moreover, the incubation was prolonged to three hours. The results of this experiment contradicted those of the two preceeding ones. Thus, when GTP was added together with ATP, the amination of IMP was intensified from 1.58 to 2.76 μmoles, while with GTP alone the amount of IMP aminated (1.32 μmoles) approached values obtained with ATP alone. In view of this disparity of results in the two sets of experiments, it was not possible to draw any definite conclusions on the participation of GTP in AMP synthesis in pigeon muscle extracts.†

Various authors who have studied the formation of AMP from IMP expressed a variety of views on the origin of the 6-amino group on the purine nucleus (in AMP). Thus Kalckar and Rittenberg [18], who based their results on the study of in vivo turnover of N^{15}-labelled metabolites in rats, considered the amide nitrogen of glutamine a possible precursor of the amino group of AMP. Kometiani et al. [8] studied IMP reamination in homogenates of animal tissues and concluded that in brain homogenates L-glutamate, γ-aminobutyrate, L-asparate and β-alanine could all serve as nitrogen donors in this reaction, while in muscle homogenates only β-alanine and L-asparate were active. Nekhorocheff [9] maintained that in frog muscle IMP could be aminated at the expense of creatine. On the other hand, the most recent investigations indicate that the most immediate precursor of AMP is a condensation product of IMP and aspartate. The specificity of this reaction was studied in the present work using the AE extracts (see "METHODS") and checking the ability of a number of compounds to substitute L-aspartate as a substrate in this reaction. The compounds tested were: D-aspartate, DL-aspartate, asparagine, L-glutamine, β-alanine and γ-aminobutyrate.



The formation of AMP from IMP and L-aspartate in pigeon breast muscle extracts, as a function of incubation time.

TABLE 2. A Comparison of the Enzymatic and Chromatographic Methods of Assay of AMP Synthesized by Pigeon Muscle Extracts. (Reaction mixtures contained: ATP, 3 μmoles; IMP, 7.5 μmoles; L-aspartate, 3 μmoles. Incubation time, one hour)

Expt. No.	ΔAMP, μmoles	
	found by method I	found by method II
1	0,75	0,73
2	0,77	0,81
3	0,61	0,69
4	1,53	1,40
5	1,52	1,42

The results of these experiments (Table 4) clearly indicate that none of the compounds tested were capable of donating an amino group to IMP to produce AMP. The DL-aspartate added was one-half as effective as L-aspartate (Table 4, Experiment 2).

The slight increase in AMP in the presence of D-aspartate could be due to the presence of a small impurity of the L-isomer in the preparation. In contrast to the results reported by Kometiani, we did not find any activity in the presence of either β-alanine or γ-aminobutyrate. The slight amount of AMP synthesis observed in the presence of L-glutamate was probably due to aspartate formation through transamination. This was also confirmed experimentally (Table 5), when glutamate was shown to be an excellent amino group donor for AMP synthesis if oxalacetate was added to the system; in those incubation mixtures the amount of AMP synthesized was about one half the value obtained in the presence of L-aspartate. It is quite possible that only part of the oxalacetate added was utilized for transamination, the remainder being decarboxylated or catabolized in a variety of pathways. The data presented

† After this paper was prepared for the press, the communication of C.L. Davey [Nature 183, 995 (1959)] came to hand. The latter author studied the reaction between IMP and aspartate in active extracts of rabbit muscle, in systems identical with those of Newton and Perry [6]. According to Davey, the enzymatic preparation contained strongly bound GTP, whose removal (by dialysis over eight days) caused complete loss of synthetic activity, which could be restored only by the addition of catalytic amounts (several hundredths of a μmole) of GTP and of purified ATP. However, the author's conclusion, based on the increase of absorption in the incubation mixture at 267 mμ, that the accumulated product of the reaction between IMP and aspartate was actually AMP is not proven. It is quite possible that the actual product was ATP, whose absorption maximum (260 mμ) is very close to that of AMP.

TABLE 3. The Effect of GTP on the Formation of AMP from IMP and Aspartate

Expt. No.	Incubation time	Additions, μmoles				NH_3 per test, μmoles	ΔNH_3 (ΔAMP), μmoles
		ATP	IMP	L-asp.	GTP		
		4,5	—	—	—	4,21	—
		4,5	10	5	—	5,31	1,10
		4,5	—	—	1	4,21	—
1	1	4,5	10	5	1	5,11	0,90
		—	—	—	1	0	—
		—	10	5	1	0,50	0,50
		3,5	—	—	—	3,33	—
		3,5	10	5	—	4,74	1,41
2	1	3,5	10	5	1	4,17	0,84
		—	10	5	1	0,82	—
		2,5	—	—	—	2,17	—
		2,5	7,5	3	—	3,75	1,58
3	3	2,5	—	—	3	2,50	0,33
		2,5	7,5	3	3	5,26	2,76
		—	—	—	3	0,26	—
		—	7,5	3	3	1,58	1,32

TABLE 4. Specificity for Amino Group Donor in AMP Synthesis from IMP in Pigeon Muscle Extracts. (ATP added—5 μmoles; IMP—7.5 μmoles)

Expt. No	Incubation time h	Additions 3 μmoles	ΔNH_3 (Δ AMP), μmoles	Expt. No.	Incubation time h	Additions, 5 μmoles	ΔNH_3 (Δ AMP), μmoles
1	2	L-aspartate	2.07			L-aspartate	1.66
		D-aspartate	0.40	4	1	L-glutamate	0.26
2	3	L-aspartate	2.35			β-alanine	0
		DL-aspartate	1.22			γ-aminobutyrate	0
		L-aspartate*	1.70			L-aspartate	1.32
3	1	L-asparagine*	0	5	1	L-glutamate	0.02
						β-alanine	0
						γ-aminobutyrate	0.27

*Amount added—5 μmoles.

TABLE 5. The Conversion of IMP to AMP in the Presence of L-Glutamate and Oxaloacetate. (Incubation time—three hours; Additions: ATP—3 μmoles, L-aspartate—3 μmoles; IMP—7.5 μmoles, L-glutamate—10 μmoles, Oxaloacetate—10 μmoles in expts. 1 and 3; 5 μmoles in expt. 2.)

Composition of mixture	Δ NH_3 (Δ AMP) in μmoles		
	expt. 1	expt. 2	expt. 3
ATP + IMP + L-aspartate	2.95	1.35	1.95
ATP + IMP + L-glutamate + oxaloacetate	1.07	0.50	1.01
ATP + IMP + L-glutamate	0.47	0	0

TABLE 6. The Effect of α-Methyl-DL--Aspartate on IMP Amination (Incubation time—1 hour; ATP added—5 μmoles, IMP—10 μmoles).

Expt. No.	Additions, μmoles		ΔNH$_3$(ΔAMP), μmoles
	L-aspartate	α-me-DL-asp.	
1	3	—	1,53
	—	100	0,47
	—	200	0,47
2	10	—	1,70
	—	100	0,23
	—	200	0,02
	—	300	0,09
	10	100	1,40
	10	400	1,50
3	10	—	0,73
	10	200	0,90
4	5	—	1,86
	5	133	1,70
	5	266	1,80
	5	400	1,70

here allow the conclusion that, in pigeon muscle as well as in other biological systems [5-7], L-aspartate is a specific donor of the amino group in AMP synthesis from IMP. These conclusions cannot be reconciled with the results of Kometiani et al. [8] or with those of Nekhorocheff and Wajzer [9].

Searching for a selective inhibitor of the process of IMP amination, we have tested the effect on AMP synthesis of α-me-DL-asp and of a number of other aspartate antimetabolites. Braunshtein et al. [19] have demonstrated earlier that α-me-DL-asp inhibited argininosuccinate formation in urea synthesis. This inhibitor was therefore considered potentially capable of inhibiting adenylosuccinate formation in the process of IMP conversion to AMP. This, however, was not the case, as shown in Table 6. AMP synthesis could not be detected in incubation mixtures where aspartate was substituted by α-me-DL-asp in concentrations greatly exceeding that of L-aspartate usually employed. On the other hand, where L- aspartate was present, the addition of a large excess of α-me-DL-asp (100-400 μmoles) produced no inhibition of AMP synthesis. It was thus apparent that α-me-DL-asp could neither participate in the reaction studied, nor did it inhibit it.

Two other structural analogs of L-aspartate were also tested under similar conditions; these were β-methyl-DL-aspartate (Table 7) and L-cysteic acid (Table 8). As in the previous instance, these two compounds, even when present in 10- to 30-fold excess over the amount of L-aspartate, failed to inhibit its condensation with IMP, and could not replace the true substrate in the reaction. In several experiments a slight increase in AMP synthesis was observed when the standard reaction mixture, containing aspartate, was also supplemented

TABLE 7. The Effect of β-Methyl-DL-Aspartate on IMP Amination. (One-hour incubation; ATP and IMP added—5 and 10 μmoles, respectively).

Expt. No.	Additions, μmoles		ΔNH$_3$(ΔAMP), μmoles
	L-aspartate	β-me-DL-asp.	
1	3	—	1,64
	3	30	1,64
	—	30	0
2	3	—	2,07
	3	60	2,17
	—	60	0

with L-cysteic acid; this phenomenon, however, was not regularly reproducible.

The ability of pigeon heart muscle extracts to catalyze AMP synthesis from IMP and L-aspartate was also studied in a small series of experiments. Under the same experimental conditions, the heart muscle extracts showed considerably lower, albeit definite, activity levels than did similar breast muscle extracts.

SUMMARY

The optimal conditions for the study of AMP synthesis from IMP without the use of isotopes have been described; the system used included ATP, L-aspartate and a pigeon muscle preparation. The sum total of adenine nucleotides was estimated by dephosphorylation with potato apyrase, deamination of the AMP formed with adenylic deaminase and measurement of the ammonia produced in the last step (Method I). Alternately (Method II) the nucleotides were estimated spectrophotometrically as AMP, following total dephosphorylation by apyrase and chromatographic purification; the values obtained by the two methods showed close agreement.

Under optimal conditions the amino nitrogen of L-aspartate was transferred quantitatively onto IMP to form AMP within three to four hours; in the presence of PGA as a phosphoryl group donor, ATP was the final product, and no AMP deamination occurred even though deaminase was present in the system. L-aspartate was shown to be specifically the only donor of amino groups capable of participating in AMP synthesis; asparagine, D-aspartate, β-alanine and γ-aminobutyrate were shown to be inactive as donors. L-Glutamate could act as a substrate only in the presence of oxalacetate, apparently due to transamination to L-aspartate under those conditions.

Three structural analogs of L-aspartate—α-methyl-DL-aspartate, β-methyl-DL-aspartate, and L-cysteic acid—were tested for their effect in the reaction studied and were shown to be incapable of either inhibiting the reaction, or replacing the true substrate.

The problem of GTP participation in the process of IMP reamination requires further study.

TABLE 8. The Effect of Cysteic Acid on IMP Amination (One-hour incubation. Additions: ATP–5 μ moles, IMP–10 μ moles)

Expt. No.	Additions, μmoles		ΔNH_3(ΔAMP), μmoles	Expt. No.	Additions, μmoles		ΔNH_3(ΔAMP), μmoles
	L-aspartate	L-cysteic ac.			L-aspartate	L-cysteic ac.	
1	3	—	1,48	3	3	—	1,52
	3	5	1,40		3	40	1,60
	—	5	0,18		3	80	1,80
2	3	—	1,55	4	3	—	1,66
	3	40	1,83		3	80	1,48
	—	40	0				

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THE EFFECT OF STREPTOMYCIN ON THE OXIDATIVE PROCESSES IN BARLEY SPROUTS

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It was established by our earlier experiments [1, 2] that the inhibition by streptomycin of the greening process is always linked with corresponding changes in oxidative processes. It was shown, in particular, that a lowering in chlorophyll content is always accompanied by an inhibition of the activity of cytochrome oxidase and polyphenol oxidase. Simultaneously, a substantial increase in the general intensity of oxygen respiration was observed. The reasons for these contradictory phenomena remained obscure.

The present work was devoted to a more detailed study of the effect of streptomycin on the respiration of plants and the separate links of the oxidative apparatus. To this end, the method of study of respiration by means of various inhibitors was employed; this is being widely used today. This method provides an understanding of the role played in respiration by the individual oxidative systems, of the changes taking place in the distribution of these systems under the effect of streptomycin, and of the factors neutralizing its effect.

Malonic acid, monoiodoacetate, azide, and cyanide are among the most studied inhibitors of respiration, and these were the ones used by us in the present investigation.

METHODS

The "Winner" variety of barley was chosen as the object of our investigation. The seeds were soaked either in a solution of streptomycin as such, or in a streptomycin solution containing Mn (55 mg/l, as $MnSO_4 \cdot 4H_2O$ or Fe (9.6 mg/l, as the ferripotassium salt of EDTA). The manganous and ferric ions served as streptomycin-inhibiting factors. The sprouted seeds were set on paraffinated gauze, with the roots dipping into corresponding solutions. The controls were soaked and transferred, after germination, to tap water. The respiration was determined manometrically in sections of barley leaves (~ 0.5 cm long) immersed in a phosphate buffer of pH 7, containing respiratory toxins in solution. Barley leaves immersed in phosphate buffer devoid of toxins served as controls.

RESULTS

Table 1 represents data which characterize the effect of inhibitors on the respiration of leaves of barley grown on various solutions. All of the inhibitors, with the exception of KCN, suppressed the absorption of O_2 in all instances, from the plants raised on water up to those grown on solutions of streptomycin, streptomycin with manganese, and streptomycin with iron. The degree of inhibition of respiratory activity was, however, different in the various setups.

It is known that malonic acid completely suppresses the activity of succinic dehydrogenase, the enzyme which catalyzes the reversible transformation in the succinic acid – fumaric acid system. In this respect, malonate is an inhibitor of the tricarboxylic acid cycle, and should therefore suppress the respiratory step which is linked with the Krebs cycle. The inhibition of respiration by malonate was observed by a number of investigators in a series of plant tissues [3-8]. The inhibitory effect of malonate is especially well pronounced at a concentration of 0.05 M in an acid medium. At pH 6 and above, the inhibitory effect of malonate may disappear, and at times even a temporary enhancement of respiration may be observed. This may be the reason for the failure of a number of investigators to observe any inhibitory effect of malonate. There is no unanimity of opinion in respect to a strictly specific effect of malonate [3, 6, 9].

Monoiodoacetate inhibits the phosphorylation of hexoses, as well as the triosephosphate dehydrogenese and alcohol dehydrogenase [10-15], e.g., it acts first on the anaerobic phase of respiration. At the same time, monoiodoacetate acts on other enzymes, although in an insignificant way [16-18]. This means that monoiodoacetate cannot be considered a strictly specific inhibitor. Its relative specificity is, however, beyond question, and therefore it can be used as an inhibitor of the anaerobic phase of respiration. Today it is known that monoiodoacetate does not act upon the oxidases directly, but it also lowers the rate of O_2 absorption [3, 4, 7, 16, 19-21]. By suppressing the anaerobic phase of respiration, it thus lowers the activity of the aerobic phase of respiration, and finally suppresses it completely.

According to our data, the effect of malonic acid and monoiodoacetate on the respiration of young plants was the same, whether they were grown on water or on streptomycin. Inhibition indicates that the systems de-

TABLE 1. Effect of Inhibitors on Respiration in Barley Leaves Grown Under Various Conditions (the mean of three experiments is reported in this and the following tables)

Inhibitors	Series 1			Series 2		
	exptl. conditions	respiration in µl absorbed O_2/g raw wt/hr	in % of controls	exptl. conditions	respiration in µl absorbed O_2/g raw wt/hr	in % of controls
Phosphate buffer, pH 7	H_2O	171,9	100	H_2O	212,4	100
Malonic acid 0.05 M		34,4	22		61,2	29
Monoiodacetate, 0.1 M		38,2	22		23,1	11
KCN_3 0.005 M		238,6	139		234,4	110
NaN_3 0.005 M		127,1	74		145,1	68
Phosphate buffer, pH 7	Streptomycin	205,5	100	Streptomycin	223,6	100
Malonic acid 0.05 M		47,5	23		65,2	29
Monoiodacetate, 0.1 M		44,5	22,0		0	—
KCN 0.05 M		292,6	142		223,8	100
NaN_3 0.005 M		200,8	98		181,1	81
Phosphate buffer, pH 7	Streptomycin	242,1	100	Streptomycin	186,2	100
Malonic acid 0.05 M		54,5	23		63,1	34
Monoiodacetate, 0.1 M		27,5	11		11,9	6
KCN 0.05 M		321,3	133		178,8	96
NaN_3 0.005 M		214,4	89		146,0	78
Phosphate buffer, pH 7	Mn	229,5	100	Fe	164,1	100
Malonic acid 0.05 M		59,7	25		64,9	40
Monoiodacetate, 0.01 M		18,3	8		11,5	7
KCN 0.05 M		278,2	121		158,4	97
NaN_3 0.005 M		174,6	76		108,2	66

pressed by these toxins play an important role in the respiratory processes. Our experiments indicate that this role is equal for the plants of the aqueous as well as the streptomycin regimens. The tricarboxylic acid and phosphorylation cycles are included in the total respiration.

While the effect of malonate and monoiodoacetate is the same for the plants of the aqueous and streptomycin regimens, the character of the azide action is entirely different. The "residual respiration", e.g., the respiration which is not suppressed by azide and which is carried out by flavonic enzymes, is considerably more active in young plants grown on streptomycin. In control plants, the "residual respiration" amounts to 74% of the total respiration, and in plants of the streptomycin regimen, 98%. This is in agreement with the results of the work of Rubin and co-workers [22-24], who established the fact that "residual respiration" is most resistant to all kinds of unfavorable influences. In young plants of the streptomycin regimen, the "residual respiration" is 24% more active as compared with the controls (on water). In the presence of ions of Mn and Fe, the activating effect of streptomycin on the "residual respiration" drops sharply. In young plants grown separately on Mn and Fe solutions, the activity of "residual respiration" is equal to that of the aqueous regimen. Thus, the activity of the enzyme-flavoproteins rises only under conditions which inhibit the metal-containing oxidases. In the regimens containing Mn and Fe, which cause a reactivation of the metalloproteids, the activating effect of streptomycin upon the flavoprotein enzymes is lowered. The azide, which is an inhibitor of metal-containing oxidases, thus prevents the effect on the flavonic enzymes from being affected by the activating influence of streptomycin.

The activating effect of streptomycin on the "residual respiration" and the total respiration was observed not only on leaf tissues, but on root tissues as well (Table 2). In order to render the effect of streptomycin more pronounced, the latter was used in this experiment in a concentration ten times higher than that used in the preceding experiments.

In a preceding paper [2] we showed that the introduction of Mn and Fe ions into the nutritive medium removed the inhibitory effect of streptomycin upon the cytochrome oxidase and polyphenol oxidase. In addition, it seemed important to determine the effect of Mn and Fe ions upon the respiration of young plants on the streptomycin regimen. The results obtained are presented in Table 3.

These data show that the effect of Mn ions upon respiration differs sharply from the effect of Fe ions. While Mn ions do not lower the respiration activity and even activate it above the effect of streptomycin as such, Fe ions markedly lower the respiration. This effect is observed in the tissues of leaves, as well as of roots. It was

TABLE 2. Effect of Streptomycin on the Intensity of Respiration and "Residual Respiration" in 11-Day-Old Barley Plants

Organs of the young plant	Regimen of the experiment	Respiration in microliters absorbed O_2/g raw weight/hr	"Residual respiration" in microliters absorbed O_2/g raw weight/hr
Leaves	H_2O	255.2	39.1
	Streptomycin, 0.1%	368.6	100.6
Roots	H_2O	405.0	39.9
	Streptomycin, 0.1%	436.0	73.6

also of interest to trace the effect of Mn and Fe ions upon other oxidizing enzymes than the cytochrome oxidase and polyphenol oxidase, which were studied by us earlier. It is known that catalase does not change its activity under the effect of streptomycin, while peroxidase is strongly activated [1]. The results obtained by us (Table 4) testify to the fact that the Mn and Fe ions also have no effect on the catalase, as is the case of streptomycin. The character of the effect of Mn and Fe ions on the activity of ascorbic acid oxidase and peroxidase is analogous to their effect on respiration. The ascorbic acid oxidase and peroxidase, which are are activated by streptomycin, do not lower their activity to the level of control specimens under the effect of Mn ions, while the ions of Fe lower the activity of both enzymes.

For a more detailed investigation of the effect of streptomycin on respiration, we conducted a study of this process by means of determining the consumption of O_2 as well as the secretion of CO_2 (Table 5). It is known that the processes of O_2 consumption and CO_2 secretion differ, depending individually on extraneous factors, without mutual interdependence.

The data of Table 5 show that the respiration is activated under the effect of streptomycin, both in respect to CO_2 excretion and O_2 consumption. The effect of Mn and Fe ions on CO_2 excretion is in general quite similar to their effect on O_2 consumption. Under these conditions, the respiratory coefficient remains practically unchanged, amounting to 1.

DISCUSSION

A detailed study of the changes in respiratory gas exchange under the effect of streptomycin shows that the increase by the latter of the total respiratory activity in young plants is achieved mainly at the expense of "residual respiration" activation. An increase in the activity of flavine enzymes is observed only where the metal-containing oxidases are being suppressed; under conditions where the latter are reactivated by means of Mn and Fe ions, the "residual respiration" is lowered.

The effect of Mn and Fe ions on the absorption of O_2 by young plants on the streptomycin regimen differs sharply from the effect of these ions on the CO_2 absorption by control plants. When Mn is introduced into the nutritive medium, the activating effect of streptomycin on the O_2 absorption is not only not lowered, but, quite to the contrary, it is enhanced.

The effect of Fe ions is always to lower streptomycin-activated respiration. This lowering is of such magnitude that plants grown in a streptomycin medium which contains Fe breathe less actively than plants on an aqueous regimen. Analogous data concerning the effect of Mn and Fe ions were obtained by us on ascorbic acid oxidase and peroxidase. This implies that the effect of Mn and Fe upon the greening process is somewhat different. Rosen [25] assumed, from his experiments on the neutralization of the inhibitory effect of streptomycin on plant growth, that in higher plants the Mn ions activate the metabolic course which is nonsensitive to streptomycin. It should be pointed out that this author did not have any experimental proof for such an assumption. The mechanism of Mn action as advanced by Rosen is quite plausible. The results of our experiments permit the assumption that the mechanism of Mn-ion action is much more complex. On one hand, Mn reactivates cytochrome oxidase and poiyphenol oxidase, thereby restoring the oxidative enzymes which are sensitive to streptomycin and are inhibited by it. Simultaneously, Mn activates the ascorbic acid oxidase and peroxidase, e. g., the enzymes which streptomycin not only fails to inhibit, but, on the contrary, activates. Our data bring out still another important peculiarity of the effect of Mn ions, namely, the inhibition of the "residual respiration", which is activated by streptomycin. Therefore, Mn normalizes the activity of the flavinic enzymes and returns it to the original level. The latter effect of Mn ions is evidently closely related to the restoration of the activity of metal-containing oxidases.

It has been known for a long time that Mn exerts a definite influence on the oxidative systems of plants. While Mn does not form an integral part of the enzyme, it is, nevertheless, an essential activator of a series of enzymatic systems (enolases, carboxylases, phosphatases,

TABLE 3. Effect of Streptomycin, Manganese, and Iron on the Respiration of 12-Day-Old Barley Plants

Organs of the young plant	Series 1			Series 2		
	regimen of the experiment	respiration in µl absorbed O_2/g raw wt/hr	in % of controls	regimen of the experiment	respiration in µl absorbed O_2/g raw wt/hr	in % of controls
Leaves	H_2O	265,0	100	H_2O	305,8	100
	Streptomycin 0,01%	355,2	134	Streptomycin 0,01%	454,4	149
	Streptomycin + Mn	386,2	146	Streptomycin + Fe	337,0	110
	Mn	319,0	120			
Roots	H_2O	321,8	100	H_2O	589,0	100
	Streptomycin 0,01%	416,4	129	Streptomycin 0,01%	721,4	123
	Streptomycin + Mn	547,2	170	Streptomycin + Fe	436,0	72
	Mn	540,8	168			

TABLE 4. Effect of Streptomycin, Manganese, and Iron on the Activity of Enzymes in 11-Day-Old Barley Plant Leaves

Enzymes	Experimental setup	Activity of the enzyme in % of control data
Catalase	H_2O	100
	Steptomycin, 0.01%	100
	Streptomycin + Mn	100
	Mn	99
	H_2O	100
	Streptomycin, 0.01%	100
	Streptomycin + Fe	100
	Fe	100
Peroxidase	H_2O	100
	Streptomycin, 0.01%	120
	Streptomycin + Mn	151
	Mn	142
	H_2O	100
	Streptomycin, 0.01%	121
	Streptomycin + Fe	114
	Fe	116
Ascorbic acid oxidase	H_2O	100
	Streptomycin, 0.01%	128
	Streptomycin + Mn	145
	Mn	177
	H_2O	100
	Streptomycin, 0.01%	129
	Streptomycin + Fe	96
	Fe	93

peptidases, etc.). Some data indicate a functional link of Mn with the peroxidase in the process of oxidation of certain substrates. Thus, dehydromaleic acid is oxidized by peroxidase only in the presence of Mn [26]. As shown by us earlier [2], both Mn and Fe neutralize the inhibitory effect of streptomycin on the synthesis of chlorophyll, as well as on cytochrome oxidase and polyphenol oxidase.

Contrary to the observations made on young plants

TABLE 5. Effect of Streptomycin, Manganese, and Iron on the Respiratory Coefficient of 12-Day-Old Barley Plant Leaves

Experimental setup	Respiration, per absorption of O_2/g raw wt per hour	Respiration, per absorption of CO_2/g raw wt per hour	$\frac{CO_2}{O_2}$
H_2O	314,0	313,4	1,0
Streptomycin, 0.01%	377,1	381,6	1,01
Streptomycin - Mn	402,6	386,2	0,96
Mn	367,4	384,4	1,05
H_2O	324,6	303,2	0,94
Streptomycin, 0.1%	391,8	375,6	0,96
Streptomycin - Fe	323,2	306,6	0,95
Fe	273,6	250,2	0,91

grown on a solution of streptomycin with Mn, where the concentration of chlorophyll and the activity of cytochrome oxidase was always lower than in plants grown on water, in young plants, grown on a solution of streptomycin with Fe, these phenomena were always more accentuated than in control plants. The results of the present work indicate that both metals lower the "residual respiration". However, unlike the Mn ions, the Fe ions essentially lower the absorption of O_2, the activity of ascorbic acid oxidase, and of peroxidase. This seems to indicate that the beneficial effect on the greening process is evidently exerted primarely via the participation of Fe in the building of the cytochrome system. By restoring the activity of the cytochrome oxidase, the iron normalizes the utilization of energy in the respiration process, which is subsequently used in the building of chlorophyll. Thus, iron is a direct constituent of the enzyme linked with the process of chlorophyll synthesis, while the beneficial effect of manganese is exerted without its direct incorporation into the enzyme molecule.

SUMMARY

It was established that the active absorption of oxygen in plants affected by streptomycin is achieved by means of enzymes of the "residual respiration" as well as, perhaps, by ascorbic acid oxidase and peroxidase. The flavinic enzymes are activated only when the metal-containing oxidases are inhibited.

The activity of the flavinic enzymes is reduced where the metalloproteins are reactivated by means of Mn and Fe ions. Mn ions do not lower the activating effect of streptomycin on oxygen consumption and on ascorbic acid oxidase and peroxidase, but, on the contrary, enhance it, while Fe lowers the oxygen consumption, as well as the activity of ascorbic acid oxidase and peroxidase. The differences between the actions of Mn and Fe ions upon the respiration and the oxidative processes causes the difference in the way they affect the greening processes.

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CHARACTERISTICS OF STRUCTURE AND PROPERTIES OF DIFFERENT ZYMOSAN PREPARATIONS

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In 1954 Pillemer and his co-workers discovered in blood a new protein , properdin, which along with all the components of complement and Mg ions,forms the properdin system and is responsible for the destruction of bacteria, binding of viruses, and lysis of some erythrocytes.

Pillemer showed that properdin can form an insoluble complex with the polysaccharides of cell membranes of yeasts called zymosan. In the destruction of this complex under definite conditions, properdin can be obtained in the free form. On this basis, a method was worked out for the titration of properdin and its preparation.

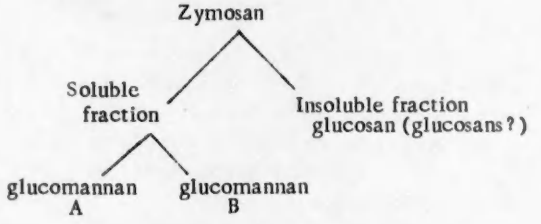
Zymosan, like many other polysaccharides of bacterial, plant, and animal origin, is not only bound with properdin in vitro, but,when it is introduced in vivo, shifts the titer of properdin in the blood [2]. The increased titer of properdin in the blood produces an increased resistance of the animals to some infections and survival after their irradiation with x-rays. The process of reaction of properdin with zymosan in experiments in vitro and in vivo, and questions of antigenic properties of zymosan and of the effect of its injection on the properdin content in the blood of animals have attracted much attention in the literature. However, questions of the composition and properties of different zymosans and of the relation between their structure and activity with respect to the properdin system have not been explained at all up to the present.

The first data on the composition of zymosan were published by Lowburg and Ricketts, who showed that zymosan does not contain glycogen and is a mixture of mannan and glucosan [3]. Di Carlo and Fiore studied the composition of different preparations of zymosan with different biological properties and found no connection between their composition and activity [4]. According to their data, the chief carbohydrate component of zymosan is glucosan (51-57%), with a considerably lower content of mannan (17-22%). In all the preparations of zymosan there was also a small amount of glucosamine (0.06-0.07%). In their work the authors used zymosans obtained by the method of Pillemer from Fleischman's yeast.

Tovarnitskii and co-workers [5] studied the acid hydrolyzate of zymosan obtained from baker's yeast and also found glucose and mannose in it.

As already reported earlier [6] we have worked out a method for fractionation of zymosan which permitted obtaining a water-soluble and an insoluble fraction of zymosan. The need for such fractionation was caused by the fact that the starting preparation of zymosan was insoluble in water, acids, alkalis, and organic solvents, which made its study especially difficult. With the help of the fractionation of zymosan and the investigation of the resulting fractions it was possible to explain that it is a complex mixture of polysaccharides with different compositions and structures.

The composition of zymosan can be illustrated by the following scheme:



Later it became known that baker's yeast is not a pure strain, but is a mixture of "cultures" and so-called "film" yeasts. Therefore it was necessary to study the composition of zymosans obtained from pure strains of yeast (strain No. 7) and to compare the domestic preparations with preparations obtained by the method of Pillemer from Fleischman's yeast (from the Fleischman laboratory). It is also of interest to compare the domestic zymosan preparations obtained by different methods [7, 8].*

* The zymosan preparations were kindly furnished to us by R.A. Rutberg (Institute of Hematology and Blood Transfusion).

METHOD OF INVESTIGATION

Fractionation of zymosan. Four g of zymosan were boiled for three hours with 25 ml of 30% KOH, after which the polysaccharide was precipitated with twice the volume of 96% alcohol and allowed to stand overnight for complete precipitation. The resulting brownish precipitate (dense, like a resin) was stirred several times with water, centrifuged, and from the combined supernatant liquids the so-called soluble fraction of zymosan was precipitated with double the volume of alcohol. Treatment of the precipitate with water was repeated until the addition of alcohol to the supernatant liquid produced no more precipitate. The rest of the precipitate, insoluble in water, was washed five or six times with water and then twice with alcohol, after which it was dried. This fraction was called insoluble. The soluble fraction was reprecipitated three or four times by alcohol from water solution, after which it was also dried. Thus we obtained a soluble and an insoluble fraction from all of the zymosar preparations which we studied.

Complete acid hydrolysis of zymosan and its fractions was carried out with 5% HCl during three hours, after which the product of hydrolysis was studied chromatographically. As the solvent we used butanol, acetic acid, and water in the ratio 4:1:5, and as the developer, aniline hydrophthalate.

Paper electrophoresis of the soluble zymosan fraction was carried out in a veronal buffer at pH 8.6 for 18-20 hours at a voltage of 140-160 v. On each paper strip we placed 20 μ liters of solution which contained 1 mg of soluble fraction. The polysaccharide on the paper strip were developed by the method described earlier [9]. The polysaccharides observed in the electrophoresis were studied by qualitative and quantitative chromatography.

Qualitative and quantitative chromatography of the electrophoretic fractions of zymosan. The electrophoretic fractions were eluted from a large number (40-50) of paper strips with hot water. The eluates were evaporated on a water bath to 1.5-2 ml, after which they were hydrolyzed with 1.5 N H_2SO_4 for two hours. The SO_4 ions were removed from the hydrolyzate with $Ba(OH)_2$. The hydrolyzates were again evaporated to minimum volume and 0.2 ml was placed on a chromatogram.

For quantitative determination of the relative sugar content on the chromatogram, only markers were developed (glucose and mannose), and from the corresponding parts of the paper, two extractions were made, with hot water, of the monosaccharides of the deposited hydrolyzate. As controls we used eluates obtained by hot-water treatment of the same size portions of the same chromatogram, but which did not contain spots. The content of sugars in the eluate was determined by the method of Hagedorn and Jensen. For calculation of the mannose content we constructed a curve of the relation of reducing power of mannose to its concentration. Hexosamines were determined by the modified method of Elson and Morgan. †

RESULTS OF THE INVESTIGATION

The study was made of four preparations of zymosan obtained from different strains of yeast by different methods, namely: zymosan No. 30, obtained from baker's yeast, by the method of Pillemer and Ecker [7, 8]; No. 34, from baker's yeast, by the modified method of Rutberg [7]; No. 1 from a pure strain of yeast, by the modified method of Rutberg [7]; and finally, zymosan obtained from yeast of the Fleischman strain, by the method of Pillemer [10].

The difference between these methods is the following. In 1941 Pillemer and Ecker [8] suggested a method of obtaining zymosan by tryptic digestion of yeast in a phosphate buffer. During the digestion, the original pH value of 8.7 fell to 6.5 because of formation of acid hydrolysis products. Therefore, to create the optimum conditions for the action of trypsin, Pillemer, in 1956 [10], suggested making the hydrolyzate alkaline as the pH fell. The modified method of Rutberg is based on the digestion of yeast in physiological saline at an acid pH of 5.4. An exception was the pure strain of yeast, in the digestion of which in physiological saline there was no appreciable acidification of the medium.

In Table 1 we give the nitrogen content in the whole zymosan preparation and in its fractions.

While the whole preparation contains from 1.4 to 3.15% nitrogen, both the soluble and the insoluble fractions contain considerably less. A small amount of it comes from the portion of hexosamine (0.04 and 0.05% for the insoluble zymosan fractions of No. 1 and Fleischman), and not from amino acids. As to the whole zymosan preparation, it evidently depends on whether digestion of the yeast was carried out under more or less optimum conditions for the action of trypsin to determine whether the preparation is obtained with less (No. 1 and No. 30) or more (No. 34) nitrogen.

The determination of the total carbohydrate content in the studied preparations gave the following results: zymosan No. 30 contained 62% carbohydrate; No. 34, 50%; No. 1, 84%; and Fleischman zymosan, 73%. It follows from these results that preparations obtained from baker's yeast (No. 34 and No. 30) contain considerably less carbohydrate (50-62%) than preparations obtained from the Fleischman strain and the pure strain of yeast (84-73%). Depending on the conditions of hydrolysis, the greatest amount of carbohydrate is present in that zymosan (No. 30) which was obtained under conditions more favorable for protein digestion. The soluble and insoluble fractions of all the zymosans are pure

† The determination of hexosamines was carried out by L.I. Linevich in the laboratory of Physiological Chemistry, Academy of Sciences, USSR.

TABLE 1. Nitrogen Content in Whole Zymosan Preparations and in Fractions (in %)

Zymosan	Whole preparation	Zymosan fraction	
		soluble	insoluble
No. 30	1,7	0,47	0,15
No. 34	3,15	0,41	0,2
No. 1	1,4	0,1	0,43
Fleischman	1,7	0,08	1,0

polysaccharides and contain 90-96% carbohydrate. Determination of the specific rotation of the soluble zymosan fraction gave the following results:

Soluble zymosan fraction	Specific rotation $[\alpha]_D$
No. 30	+105°
No. 34	+131°
No. 1	+114°
Fleischman	+ 76°

These results show that the specific rotation distinguishes the soluble fraction of the Fleischman zymosan preparation very markedly from the others.

Chromatographic analysis of the products of complete acid hydrolysis of zymosan allowed us to observe the presence only of glucose and mannose in all the preparations, which agreed with the data in the literature [3-5]. The soluble fraction of all the preparations also consisted of glucose and mannose, while in the insoluble fraction, only glucose was found. Thus, the insoluble zymosan fraction is a glucosan. The soluble fractions of all the preparations were studied by paper electrophoresis (Figure).

As is shown by the electrophoretograms, the soluble fraction of zymosans No. 30 and No. 34, obtained from commercial baker's yeast, consists of two fractions, A and B, which differ in their electrophoretic mobility. The products of the oxidation of these fractions by periodic acid are colored differently by the Schiff reagent. Fraction A (mobile) becomes violet, and fraction B (nonmobile) becomes blue. The soluble fraction of the zymosan obtained from yeast of pure strain No. 7 and the Fleischman strain consists of one nonmobile fraction B. It may be that fraction A is typical of the "film" yeasts which, as already mentioned, are found in great quantity in baker's yeast in the form of an impurity; therefore, pure yeast strains do not contain this fraction. Qualitative chromatographic analysis of fractions A and B showed that both fractions consist of glucose and mannose, and hence are glucomannans a and b (corresponding to fractions A and B). Quantitative determination of the ratio of both components in glucomannan is given in Table 2.

Table 2 shows that glucomannan b for each strain of yeast is characterized by a definite ratio of glucose

TABLE 2. Ratio of Glucose to Mannose in Glucomannans a and b in Different Zymosans. The figures given are the average of three experiments.

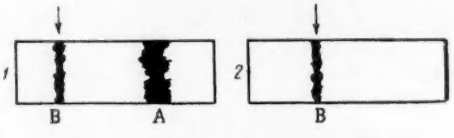
Zymosan	Glucomannan a (mobile)	Glucomannan b (nonmobile)
No.30	1:1.6	1.6:1
No.34	1:1.2	1.6:1
No.1	None	1:1
Fleisch-man	None	2:1

and mannose (1.6:1; 1:1; 2:1), which is evidently specific for each strain. The soluble fractions of zymosans obtained from baker's yeast (No. 30 and No. 34) have an identical glucomannan b and different glucomannans a. The latter differences obviously are determined by the different methods of obtaining these preparations, but the explanation of the dependence of structure of the glucomannans a on methods of obtaining the preparations is not possible at the present time. The considerable variation in ratios of glucose and mannose in the different glucomannans should be noted (Table 2). Similar facts have often been noted in the literature for glucomannans of different origins. Thus, glucomannans have been described in which the ratio of glucose to mannose was 1:2.5 [11], 1:3 [12], and 1:2 [13]. Evidently the glucomannans of yeast are not unique in this respect.

The soluble zymosan fractions are not decomposed by blood plasma and serum, nor by extracts from rabbit livers at different pH values. Evidently animal tissues lack the enzyme systems which participate in the splitting of glucomannans. The determination of the biological activity of the resulting fractions toward the properdin system showed that the soluble fractions did not have biological activity in vitro, while the insoluble fractions under these conditions combined with properdin.

DISCUSSION

Our results on the fractionation of the original zymosan showed that there are several different poly-



Electrophoretograms of the soluble fractions of different zymosans. 1) Zymosans No. 30 and No. 34; 2) zymosan No.1 and Fleischman. A and B, zymosan fractions. Arrow indicates point of introducing the solution.

saccharides in its composition: glucosans and glucomannans.

The glucomannans are distinguished from each other by the ratios between glucose and mannose. Evidently, the ratio is characteristic for each strain of yeast. In the case of baker's yeast, which contains an admixture of the film forms, the soluble zymosan fraction contains two different glucomannans which can be separated by paper electrophoresis. According to the literature, zymosans contain glucose and mannose. These results were based on the study of the products of complete acid hydrolysis of zymosan. The separation of the soluble fractions from zymosan permits demonstration in them, using paper electrophoresis, of glucomannans, and the separation of these from glucosans. The fact that the polysaccharides of the cell membranes of yeasts consist of glucosans and two different glucomannans was shown somewhat earlier and independently of us by Kessler and Nickerson [14]. They obtained the glucomannans in the form of the complexes glucomannan-protein I and glucomannan-protein II after suspending the yeast cell membranes in 1 N KOH and treating the alkaline hydrolyzate with ammonium sulfate. The amino acid composition and ratio of glucose and mannose in the two complexes differed.

The electrophoretically homogeneous glucomannans in our experiments with alcoholic fractionation (precipitation by equal and by triple volumes of alcohol) did not differ in their components. This gives us reason to suppose that the zymosans do not contain mannans. It was shown that the glucomannans do not have biological activity toward the properdin system. According to the data of Pillemer, yeast glucosans bind properdin but do not inactivate C_3^1 [2]. It was noted that the insoluble zymosan fraction (glucosan) has the ability to bind properdin. Thus, the biological activity of zymosan toward the properdin system is evidently related to its glucosan fraction.

Since the starting zymosan preparation contains a considerable amount of protein nitrogen, and the insoluble fraction does not contain protein nitrogen, the latter could have considerable advantage in experiments in vivo. Therefore it is an important and promising problem to obtain the most active possible insoluble zymosan fraction.

SUMMARY

In obtaining zymosan by different methods, depending on the conditions of digesting the yeast, the content of nitrogen in the preparations varies from 1.4 to 3.15%. Zymosans obtained from baker's yeast contain considerably less carbohydrate (50-62%) than do zymosans obtained from pure strains (73-84%).

We have worked out a method for fractionation of zymosan which permits obtaining soluble and insoluble fractions which contain almost no nitrogen. The content of carbohydrates in these fractions is 90-96%. The soluble zymosan fraction consists of glucose and mannose, and the insoluble one, only of glucose.

The composition of the different zymosans depends on the strain of yeast. Zymosans obtained from pure strains (No. 7 and Fleischman) do not contain an electrophoretically mobile glucomannan a, which is found in preparations obtained from baker's yeast. Evidently glucomannan a is specific for film yeasts. Glucomannan b of different zymosans differs in the ratio of glucose and mannose.

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THE ELECTROCHEMICAL PROPERTIES OF IMMUNE PROTEINS SYNTHESIZED IN AN IRRADIATED ORGANISM

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According to the results of a number of authors [1, 2] antibodies against bacteria of the typhoid group are localized in the rapidly migrating fraction of serum γ-globulin (fractions γ_1 or T). At the same time it has been established that agglutinins against Flexner dysentery bacteria migrate in the electric field with the α_2-globulins [3].

In a previous communication [4] we showed that in rabbits injured by x-rays at definite doses there occurred a sharp suppression of the synthesis of precipitins and nonspecific γ-globulins and a simultaneous activation of the production of antiparatyphoid agglutinins, and also of α- and β-globulin fractions. Considering that under physiological conditions with repeated immunizations the antibody can change its localization in the protein fractions of blood, shifting toward the highly dispersed fraction [5], we felt it of interest to compare the dynamics of change in electrochemical properties of different forms of the antibody produced in healthy organisms and those injured by ionizing radiation.

METHODS

As experimental animals we used rabbits weighing 1.8-2.2 kg, which before immunization gave a negative reaction for agglutination and precipitation in dilutions of 1:5. Irradiation was carried out in an RUM-3 x-ray apparatus. Conditions of irradiation: 180 kv, 10 ma, skin-focus distance 40 cm, filter 0.5 mm Cu, force of dose 30 r/min, without a tube. Total dose, 600 or 1000 r.

Immunization was begun 24 hours after irradiation. The rabbits were injected subcutaneously three times at ten-day intervals with heated vaccine of Bact. paratyphi B. (in doses of 500 million, 1 billion, 1 billion microbe bodies) and the blood serum of human donors (in doses of 0.5, 1, 1 ml). Study was carried out on the tenth day after each immunization. Blood was taken from the marginal vein of the ear in amounts of 12-15 ml. The amount of antibody was determined by specific serological reactions of agglutination and precipitation.

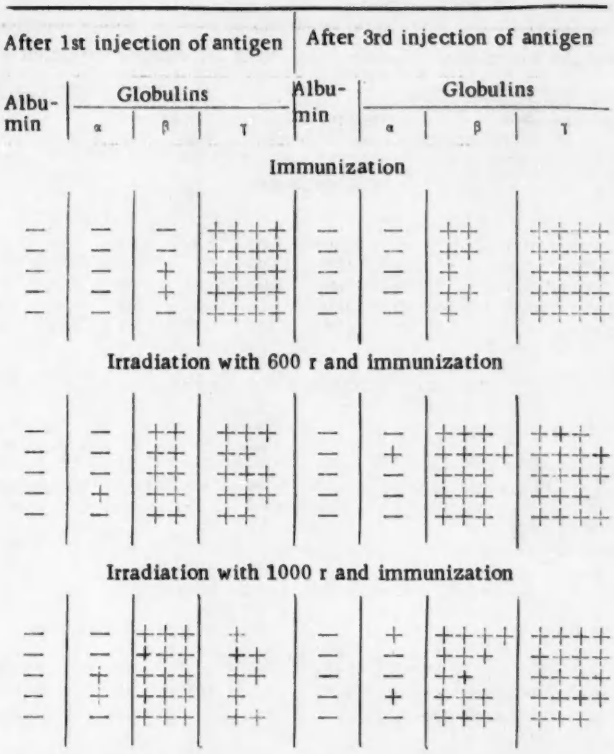
Localization of the agglutinin was established by electrophoresis of serum proteins in combination with serological control of the separate fractions. For this purpose 0.02 ml of rabbit serum, free from hemolysis, was submitted to paper electrophoretic separation in a veronal-medinal buffer (pH 8.6, μ 0.1) for three hours with a potential gradient of 16 v/cm. One of two parallel electrophoretograms was dried at 90° for 30 min and developed with bromophenol blue. On the colored strip we noted the zones of separate protein fractions which occurred on the uncolored electrophoretogram containing the native protein.

The portions which corresponded to albumin and the different globulin fractions were cut out, cut to pieces, and each fraction was eluted with 1.0 ml of 0.85% NaCl for 30 min. In this way we obtained a 50-fold dilution of each protein fraction contained in 1 ml of whole serum. We set up an agglutination reaction with each eluate and with its dilution of 2, 4, and 8 times. Determination of the electrophoretic mobility of the precipitin was carried out by an electrophoresis-precipitate method [6].

The results were controlled by fractionation of the serum proteins with $(NH_4)_2SO_4$ with later electrophoresis of the serologically active fractions. To the twice-diluted serum at 20° was added dropwise with constant stirring the required amount [7] of a saturated solution of $(NH_4)_2SO_4$. Fractional precipitation of the serum proteins was carried out between the limits of 15 to 45% saturation, increasing the concentration each time by 5%. The precipitated protein was separated by centrifuging for 15 min at 1500-2000 rpm. The supernatant liquid was dialyzed in a cellophane bag against physiological saline and the end of the dialysis was determined by a negative reaction for SO_4 ion in the supernatant liquid dialyzate; taking the dilution into account, we ran a quantitative reaction of agglutination of precipitation.

The serologically active precipitate was dissolved in physiological saline and placed in a cellophane bag for dialysis, which was carried out against distilled water.

TABLE 1. Electrophoretic Mobility of Agglutinins Synthesized in the Organisms of Healthy and Irradiated Rabbits



Note. ++++ means a positive reaction of agglutination with the eluate diluted 8 times; +++ means reaction with the eluate diluted 4 times; ++ means reaction with the eluate diluted 2 times; + means reaction with whole eluate; minus means negative reaction with whole eluate.

Again the precipitated protein was placed in a centrifuge tube and separated by centrifuging, after which it was dissolved in 1.0 ml of 0.85% NaCl. The solution thus obtained of the protein fractions which contained antibodies was submitted to electrophoretic study.

RESULTS

Determination of electrophoretic mobility of agglutinins. The experiments were carried out on rabbits separated into three groups: immunized with vaccine of B. paratyphi B.; irradiated with a dose of 600 r and immunized; irradiated with a dose of 1000 r and immunized.

In the group irradiated with a dose of 600 r, the rabbits, after the first and after the second and third antigen injections, showed localization of the agglutinin in the β- and γ-globulin fractions of serum. In healthy immunized animals, after the first injection of the antigen, the antibody was determined to have an electrophoretic mobility equal to that of nonspecific γ-globulin (Table 1). The slight agglutinating power which appeared in the β-globulin fraction of two rabbits must evidently be considered an artefact caused by the less perfect separation of β- and γ-globulins in some cases.

Successive injections of antigen caused, in three out of five control animals, the appearance of agglutinins with the electrophoretic mobility of β-globulins. A contradiction to this picture was found in rabbits irradiated with a dose of 1000 r. After the first injection of antigen, most of the antibodies in the rabbits migrated with the β-globulin fraction, the eluate of which had maximum agglutinating power. Considerably less serological activity was possessed by the γ-globulin fraction, and still less by the α_2-globulin fraction. The second and, especially, the third antigen injections caused the appearance of a large amount of antigen with the electrophoretic mobility of the γ-globulins.

Similar results were obtained by using the method of fractional salting out of serum proteins (Table 2). As this table shows, at 15% saturation of the serum with $(NH_4)_2SO_4$, in all the animals there was formed a preci-

TABLE 2. Serological Activity of Different Protein Fractions from Rabbit Blood Serum after Immunization with B. parathyphi B.

After 1st antigen injection								After 3rd antigen injection							
serum	15%	20%	25%	30%	35%	40%	45%	serum	15%	20%	25%	30%	35%	40%	45%
	γ	γ	γ	γ	β	β/α_2	α_2		γ	γ	γ	γ	β	β/α	α_2
Immunization															
320	320	240	240	0	0	0	0	2560	2560	1920	1280	640	320	0	0
640	640	320	240	0	0	0	0	1280	1280	960	960	480	160	0	0
640	640	320	320	0	0	0	0	2560	2560	1280	320	160	0	0	0
320	320	240	160	0	0	0	0	2560	2560	1920	960	480	320	0	0
640	640	480	240	0	0	0	0	1280	1280	960	320	160	60	0	0
Irradiation with 600 r and immunization															
160	160	120	60	20	20	0	0	640	640	480	320	120	60	0	0
120	120	80	80	40	20	0	0	1280	1280	960	960	320	80	20	0
160	160	120	60	40	20	0	0	1280	1280	960	480	160	40	0	0
320	320	160	120	80	30	0	0	960	960	640	480	120	80	0	0
120	120	80	40	30	30	0	0	1280	1280	960	640	320	60	0	0
Irradiation with 1000 r and immunization															
160	160	160	120	120	80	20	0	2560	2560	1920	960	480	320	60	0
160	160	120	80	60	40	20	0	2560	2560	1280	960	960	480	80	0
320	320	240	240	80	60	30	0	1280	1280	960	480	320	120	60	0
240	240	240	160	120	80	40	0	2560	2560	1920	640	320	160	80	0
240	240	240	160	80	60	20	0	1280	1280	960	480	160	80	20	0

Note. In this and the following table the figures in the second line above indicate the percent saturation of the serum with ammonium sulfate; the figures in the remaining lines, the dilution of the supernatant liquid which gives a positive agglutination reaction.

pitate which did not contain antibodies, since the titer of the supernatant liquid was then not changed. The precipitation of serologically active proteins in rabbits of the first and second groups began at 20% saturation of the serum with $(NH_4)_2SO_4$, and, in rabbits of the third group, at 25% saturation.

After the first antigen injection in the control animals, complete salting out of the agglutinins was attained as early as 30% saturation, while after the third antigen injection, not until 40%. On irradiation with 600r after all three immunizations, the serologically active globulins precipitated in the range from 20 to 40% saturation with $(NH_4)_2SO_4$. In rabbits injured lethally by a dose of 1000 r, after the first antigen injection the antibodies were salted out in the range from 25 to 45% saturation, and after the second and, especially, after the third injection, in the range from 20 to 45%. The results of electrophoretic control of the serologically active protein fractions are given in Table 2.

It was shown that in the interval from 20 to 30%, the γ-globulins were salted out; in the interval from 20-40%, the γ-, β-, and an initial portion of α_2-globulins, and, finally, in the interval from 25 to 45%, the highly mobile portion of the γ-globulins, and also the β- and α_2-globulins were salted out.

Determination of the electrophoretic mobility of precipitins. The experiments were carried out on 15 rabbits, divided into three groups: immunized by serum donors; irradiated with a dose of 600 r and immunized; irradiated with a dose of 1000 r and immunized. The results of the experiments are given in Table 3.

By fractional salting out with $(NH_4)_2SO_4$ it was shown that the serologically active globulins after the first antigen injection are salted out in all three groups of animals in the range from 20 to 25-30% saturation with electrolyte, which corresponds electrophoretically to serum γ-globulin. After the third injection in the blood of the control animals, precipitins appear which have an electrophoretic mobility of β-globulins, being salted out in the range from 30 to 35%. At the same time, in irradiated animals, the precipitins, as before, remain in the γ-globulin fraction (Table 3).

TABLE 3. Serologically Active Protein Fractions of the Blood Serum of Rabbits Immunized by Serum Donors

After 1st antigen injection								After 3rd antigen injection							
serum	15%	20%	25%	30%	35%	40%	45%	serum	15%	20%	25%	30%	35%	40%	45%
Immunization															
400	400	300	75	0	0	0	0	6400	6400	4800	1600	800	0	0	0
400	400	300	50	0	0	0	0	4800	4800	3200	2400	1200	0	0	0
300	300	100	50	0	0	0	0	6400	6400	3200	1200	0	0	0	0
400	400	150	100	0	0	0	0	3200	3200	1600	600	300	0	0	0
600	600	300	150	0	0	0	0	4800	4800	2400	1600	400	0	0	0
Irradiation with 600 r and immunization															
50	50	25	10	0	0	0	0	300	300	75	0	0	0	0	0
25	25	10	0	0	0	0	0	200	200	75	0	0	0	0	0
25	25	10	0	0	0	0	0	200	200	100	75	0	0	0	0
100	100	75	25	0	0	0	0	100	100	50	25	0	0	0	0
75	75	50	50	0	0	0	0	150	150	75	0	0	0	0	0
Irradiation with 1000 r and immunization															
25	25	10	10	0	0	0	0	150	150	75	25	0	0	0	0
25	25	10	0	0	0	0	0	100	100	50	0	0	0	0	0
50	50	10	10	0	0	0	0	300	300	200	50	0	0	0	0
25	25	25	10	0	0	0	0	100	100	25	0	0	0	0	0
50	50	10	0	0	0	0	0	100	100	50	0	0	0	0	0

The results obtained with the aid of the electrophoresis-precipitation method of Gurvich [6] also showed the presence of precipitins in both healthy and irradiated animals in the zone which corresponds to serum γ-globulin.

DISCUSSION

These investigations show that in radiation sickness there is a change in the molecular weight and value of the electrophoretic potential (factors which determine the electrophoretic mobility of colloidal particles) of the antiparatyphoid agglutinins. There are suggestions in the literature of the dependence of the physicochemical properties of immune globulins on the share of different organs in the process of their formation [8]. It is also known that different sections of the reticuloendothelial system share unequally in the production of globulins which have different rates of migration in the electrical field [9].

The synthesis of the α- and β-globulin fractions, activated by radiation injury [10], is carried out in the relatively radioresistant Kupffer cells of the liver [11, 12]. There is evidence in the literature of the possibility of participation of the liver in the synthesis of agglutinins against Bact. paracoli and B. paratyphi B. [13]. In radiation sickness there occurs, in the liver cells, an accumulation of ribonucleic acid [14] which is of great value in the processes of protein synthesis [15]. According to the ideas of Burnet [16], the formation of antibodies occurs in two steps, first in the reticuloendothelial cells; second, in the lymphocytes and plasmocytes.

In view of this, we are inclined to consider that the shift of agglutinins toward the more highly dispersed fractions of serum proteins is related to a change in the site of the main synthesis of antibodies in damage to the organism by penetrating radiation. It is possible that because of severe degenerative-necrotic changes caused by ionizing radiation in the lymphocytes and plasmatic cells, the process of formation of antibody molecules from beginning to end occurs in the more radioresistant Kupffer cells of the liver. The latter apparently also take part in the production of antibodies in the healthy rabbit, but only during the period of high intensity of this process, after the third injection of antigen, when the immunological "potential" of the regional lymph nodes is exhausted [17]. Naturally, in answer to stimulation by the antigen, the liver synthesizes antibodies which have the electrophoretic mobility of β-globulins. By the dependence of the electrophoretic mobility of the antiparatyphoid agglutinins on the site of their production, we can also explain the appearance of antibodies in the γ-globulins of rabbits irradiated with a dose of 1000 r in the period of restoration of functional activity of the lymphatic tissues (30 days after irradiation). The

synthesis of precipitins against human serum, which does not change its localization in the γ-globulins, occurs almost entirely in the extrahepatic portion of the reticuloendothelial system. This,therefore,also explains the difference in degree of suppression of the production of agglutinins and precipitins which we observed previously [4].

The different relation of the Kupffer cells to the corpuscular and soluble antibodies can be considered the result of a high threshold of their stimulation, so that only the corpuscular antibodies are immunologically effective. However, we do not consider this idea as the only probable one. The possibility is not excluded that the change in electrophoretic mobilty of the agglutinins is due to destruction of the normal processes of their biosynthesis in the lymphoid elements.

SUMMARY

Agglutinins against Bact. paratyphi B. and precipitins against the proteins of human serum,which are produced in the healthy rabbit after the first injection of antigen,migrate in the electric field along with the γ-globulins. A third injection causes the appearance in the blood of the animals of an antibody with the electrophoretic mobility of β-globulins.

In the organism of rabbits irradiated with doses of 600 r, during the whole period of study we found synthesis of antiparatyphoid agglutinins with the electrophoretic mobility of γ- and β-globulins.

After the first injection of antigen in animals irradiated with a dose of 1000 r, agglutinins appeared in their blood which were localized in the γ-, β_1-, and α_2-globulin fractions. The third injection of antigens caused formation of antibodies with the mobility of γ-globulins. Precipitins in the injured rabbits were localized only in the γ-globulin fraction.

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THE PLUTONIUM CONTENT IN THE PROTEIN FRACTION OF RAT TUBULAR BONES

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Many animal experiments have shown that when plutonium enters the organism in large amounts, it is stored up in the bones and is retained in them for a long time [1-3].

In order to explain the reason for the strong fixation of this element in the bone tissue, we must know the character of its distribution in the separate bone fractions. However, detailed information on this question is lacking in the literature, and there are only a number of papers on the deposition of plutonium in the organic matrix of bone [4, 5].

In the present work we give the results on the content of plutonium in various fractions of rat tubular bone.

METHODS

The experiments were carried out on 68 white rats (males) weighing 160-180 g. Plutonium was injected intraperitoneally into the animals in the form of a solution of the citrate complex (pH 6.0) in the amount of 1.9 μcurie per rat. The animal was killed 7-14 days after injecting the radioactive element. The diaphyses of tubular bones were freed from muscle and the bone marrow was scraped off. In some of the experiments the powdered bone tissue (40 mesh) was twice treated with cold 5% TCA for 10 min to separate the acid soluble substances. The remaining portion was defatted with alcohol and ether. In most of the experiments the bone was decalcified by the method of Ebner [6], defatted with alcohol and ether, and the remaining tissue was ground into a powder. For separation of the organic matrix, a 100-200-mg sample of the bone powder treated in this way was taken and further treated by one of the methods given below. According to the method of Stacy [7] the organic matrix of bone tissue was separated by autoclaving (pressure 1.5 atm, temperature 120°) into the following fractions: 1) protein dissolved in autoclaving not precipitated by TCA (collagen); 2) protein dissolving in autoclaving, but precipitated by TCA; 3) protein which did not dissolve in autoclaving.

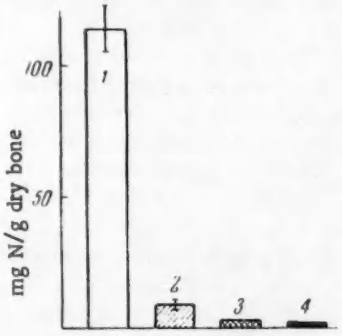

Fig. 1. Content of various proteins in rat bone. 1) Collagen; 2) residual protein; 3) albumin; 4) mucoids.

The plutonium content in the protein fractions isolated by this method varied within wide limits. Assuming that the chief difference between the results of separate experiments could be due to the severity of the conditions of treating the bone tissue (autoclaving at 1.5 atm and 120°), we tried to carry out further separations of the proteins of bone substance without autoclaving, using the methods of Eastoe [8] and Slack-Neuberger [9]. The method of Eastoe permitted separate extraction of proteins of the type of albumin and globulin (this fraction from now on is called "albumoid") and the mucopolysaccharide protein complex (mucoid). Collagen and the remaining protein were found in the precipitate. In treatment of the bone according to Slack-Neuberger, all the noncollagen protein was hydrolyzed by trypsin; collagen was in the residue; the process of autoclaving was thus excluded.

In connection with the fact that the above-mentioned processes did not permit getting all the protein fractions separately without autoclaving, in a number of experiments the bone was treated by the following method, which is a modification of the lost two methods.

Decalcified bone powder was treated successively with 10% NaCl and a half-saturated solution of $Ca(OH)_2$, thus isolating the albumoid and mucoid. The remaining substrate was submitted to hydrolysis by trypsin and gave two more fractions of protein: residual protein in solution and collagen as a precipitate. In the isolation of the protein fractions we determined nitrogen by the micro-Kjeldahl method.

For determination of the plutonium content, fractions were ignited in concentrated nitric acid with perhydrol to a white residue. The residue was dissolved in 1.5 N HNO_3 and 1 ml of the solution was placed on a target. Measurement of the activity was carried out on an ap-

TABLE 1. Plutonium Content in the Mineral and Organic Portions of Rat Bone Tissue in % of the amount of Pu in undecalcified bone

Method of treating the bone	No. of experiments	Mineral part	Organic part
Short treatment with cold TCA	5	4.5 ± 0.4	87.5 ± 2.0
Decalcification by Ebner method	17	6.0 ± 0.4	–

TABLE 2. Plutonium Content in Bone Protein (in% of the amount of Pu in decalcified air-dried powder)

Method of separation	No. of experiments	Albumoids	Mucoids	Residual protein	Collagen
Eastoe	8	14,5±0,9	4,4±1,0	64,8±4,3	
Slack-Neuberger	6	16,1±2,2			81,4±2,4
Combined method	5	15,5±2,1	3,1±0,6	5,4±1,2	65,1±5,7

TABLE 3. Relative Specific Activity* of Protein Fraction of Rat Bone

Method of separation	No. of expts.	Albumoids	Mucoids	Residual protein	Collagen
Eastoe	8	5,37±0,36	2,13±0,46	0,49±0,03	
Neuberger-Slack	6	1,12±0,11			0,63±0,02
Combined method	5	5,86±0,77	1,46±0,29	0,51±0,05	0,58±0,13

* $RSA = \frac{\text{Pu/mg N of fraction (disintegrations/min)}}{\text{Pu/g dry bone (disintegrations/min)}} \times 100.$

paratus of type D-50. All the results were treated statistically and are given in the form of mean values with the mean error.

RESULTS

In Table 1 we give data on the Pu content in the mineral and organic parts of rat bone tissue. The data of the table show that the greatest amount of the radioactive element is bound to the bone protein. Both TCA treatment and decalcification by the Ebner method indicate not more than 6% Pu in the mineral part of the bone tissue, and the rest of the amount is isolated along with the proteins.

Further study was directed to the question of how Pu is distributed among the different protein compounds of the organic matrix. The results of these experiments are given in Tables 2 and 3. The data of Table 2 show that 65-80% is fixed in collagen, 15% in albumoid, 3-4% in mucoid, and about 5% in the remaining protein, the percentages being expressed in terms of the amount of Pu in dry, decalcified bone. However, the high content of Pu found in collagen is not evidence of a great affinity of Pu for collagen, since the content of collagen in bone is ten times greater than the sum of the non-collagen proteins.

As the figure shows, collagen makes up 90% of all the protein; albumoids, mucoids, and residual protein account respectively for 2.0, 1.6, and 6.9% of the nitrogen. Conclusions as to the preferential binding of Pu with one or another protein fraction can be based on referring the data to the amount of radioactive isotope in these fractions per unit weight of nitrogen.

In Table 3 we give the values of the relative specific acitivity (RSA) of the proteins calculated per mg N of the protein fraction.

The albumoid fraction is the most active. The RSA of this fraction is ten times higher than the corresponding value for collagen and residual protein and 4 times higher than for mucoids. The explanation for this must probably be sought in the much greater active metabolism of the proteins which make up the albumoid fraction. Since the protein portion of the mucopolysaccharide protein

complex is collagen, we would expect the same value for the RSA of this fraction. However, the RSA of the mucoids is 2.5 times greater than that of collagen. Therefore we can assume that Pu is bound not only with the protein component of the mucoid molecule, but evidently also with the SO_{4--} group of chondroitin sulfate which shares in fixation of Pu.

A number of authors [10, 11] have explained the uptake of cations (Na^+, Ba^{++}) in the organic matrix of bone only by their combination with the sulfate group of chondroitin sulfuric acid. In the case of Pu, as our experiments show, the binding of the radioactive element is not limited to the sulfate group. Pu is found in all the protein fractions of bone tissue, and the mucoid binds only 4%. Probably Pu is bound chiefly to the carboxyl groups of the protein molecules, but the stability of this compound is determined by the presence of other active groups of the protein (amino groups, hydroxyl groups). As an example of such a compound of protein and metal we have the well-known complex of collagen with chromium [12, 13]. It is not impossible that adsorption of the radioactive element on the protein is of definite significance in the mechanism of fixation of Pu by bone tissue.

The results of our experiments give us reason to suppose that one of the causes for the long retention of Pu on bone is the small rate of metabolism of collagen, with which 65-80% of the radioactive element is bound.

SUMMARY

We have studied the nature of the distribution of plutonium in the various fractions of the diaphysis of tubular bone. The major part of the radioelement is fixed in the organic matrix (up to 90%).

From the organic substances of bone tissue we have isolated four protein compounds: collagen, albumoid, mucoid, and residual protein. Collagen makes up 90% of the bone protein. Albumoids, mucoids, and residual protein make up respectively 2.0, 1.6, and 6.9% (based on nitrogen). All the bone proteins contain plutonium. With collagen was bound 65-80%; with albumoid, 15%; with mucoid, 3.4%; and with residual protein, 5% of the plutonium. The metabolically active protein (albumoid) binds 10 times more plutonium per mg of nitrogen than do collagen and residual protein. The relative specific activity of mucoid surpasses by 2.5 times that of collagen, which is probably explained by a share of the SO_4^{--} group of chondroitin sulfuric acid in the fixation of plutonium.

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A STUDY OF THE CARBONYL FRACTION FROM THE HYDROLYZATE OF THE WATER SOLUBLE PIGMENT COMPLEX FORMED BY THE BRACKET CHAGI FUNGUS

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In the study of the chemical composition of chagi it was shown that the excrescence of sterile mycelia of this fungus contains up to 50% of an amorphous, dark-colored, water-soluble product which is easily precipitated from water solution by adding to the latter a mineral acid and salt. In the hydrolysis with HCl under pressure, this pigment complex is split into a number of aromatic compounds. We have isolated from the hydrolysis products in pure crystalline form three aromatic acids which we have identified: syringic, vanillic, and p-hydroxybenzoic [1]. Besides these acids we have observed in the hydrolyzate the presence of substances with an intense dark brown fluorescence whose nature has not been studied.

The purpose of the present work is the study of the composition of these compounds and an explanation of their chemical nature. For the solution of this problem we have used the method of paper partition chromatography and chromatography on a column of silica gel.

Originally the components with the dark brown fluorescence were observed in the acid fraction of the hydrolyzate from the chagi pigment complex. It was later shown that these compounds contain a carbonyl group.

In view of this, in the present paper we give the results of a study of the carbonyl fraction of the hydrolyzate which was carried out by treatment of the reaction mixture with a solution of sodium bisulfite.

EXPERIMENTAL AND DISCUSSION

Obtaining the carbonyl fraction from the hydrolyzate of the chagi pigment complex. The water solution of chagi was acidified with HCl to a pH value of about 2-3. The dark brown precipitate which separated was filtered off, washed with water, and hydrolyzed for one hour with 12% HCl in an autoclave under a pressure of 1.5 atm. After hydrolysis, the reaction mixture was carefully extracted with ether. The resulting ether solution was treated several times with 20% sodium bisulfite to bind the carbonyl compounds; the bisulfite solution, which contained the carbonyl compounds, was then carefully decomposed with dilute HCl (1:1), the excess SO_2 was removed in a vacuum at room temperature, and the liberated carbonyl compounds were extracted with ether in one series of experiments and with benzene in another. The ether solution was intensely yellow, the benzene solution was greenish yellow.

Both solutions gave reactions characteristic for the aldehyde group. The total amount of carbonyl compounds present in the bisulfite fraction was determined by precipitation with 2,4-dinitrophenylhydrazine by the method of Creighton and co-workers [2]. For this purpose, the bisulfite fraction after removal of the SO_2 was diluted with distilled water to 300-500 ml, then corresponding samples of 30 or 50 ml were taken and to them was added a hydrochloric acid solution of 2,4-dinitrophenylhydrazine (2,4-DNPH). The precipitated hydrazone was filtered off, washed, dried in a vacuum desiccator, and weighed. The total amount of substance soluble in organic solvents was determined by removal of the solvent and weighing the residue.

The results of the analyses show that the bisulfite fraction contains 30-45 % of the dry substance in the original ether sample. The content of carbonyl compounds determined by 2,4-DNPH varied in separate samples from 18-35% with respect to the dry substances of the original ether sample.

Study of the bisulfite fraction by microchromatography. The composition of the carbonyl fraction was studied by partition chromatography on strips of filter paper using the system of organic solvents recommended in the literature for the separation of mixtures of aromatic aldehydes [3-7]. At the same time we used the system chloroform-acetic acid-water (50:25:25), which we had used for study of the acid fraction of chagi hydrolyzate. Good results were obtained with the solvent ligroin-acetic acid-water (50:25:25) and water-acetic acid-ligroin - chloroform-methanol (25:25:70:20:10). The chromatograms were developed, depending on the experiments, with the following reagents: diazotized

R_f Values and Color of the Spots of the Bisulfite Fraction (Benzene Solution) with Various Reagents

Spot no.	R_f	Spot color				Fluorescence
		with phloroglucinol	with sulfanilic acid	with p-nitroaniline	with 2,3-DPNH	
1	0,60—0,70	First rose, then violet	—	—	—	Dull violet
2	~0,85	Pale rose	Rose	Dull bluish	Orange	Violet
3	0,90—0,95	Intense red	Orange	Dull greenish-blue	Light yellow	Dark brown

solutions of sulfanilic acid and p-nitroaniline, hydrochloric acid solution of phloroglucinol, and hydrochloric acid solution of 2,4-DNPH. All the chromatograms were viewed in ultraviolet light.

The results of the experiments showed that the products soluble in the bisulfite fraction were more completely extracted by ether than by benzene. The composition of the ether extract was complex: We found 10-12 spots on the chromatogram which differed greatly from each other in size and color of their reaction with the developing reagents; in all the chromatograms two spots immediately attracted attention: a large greenish yellow one which fluoresced dark brown in ultraviolet light, and a somewhat less greenish one which fluoresced a dull violet. The compounds which gave these spots on the chromatogram were thus the chief components of the bisulfite fraction. In treating the bisulfite benzene solution these compounds passed completely into the benzene layer, while most of the other components remained in the water layer and were extracted from the latter by ether.

In further work we studied only that part of the bisulfite extract which was extracted by benzene. In chromatography of the benzene extract with the solvent chloroform-acetic acid-water, three spots developed on the chromatogram.

In the table we give the R_f values for these spots with the solvent mentioned, and the colors of the spots with different developing reagents.

Spot 3, greenish yellow on the chromatogram and fluorescing dark brown, is much larger than the other spots. It should be particularly noted that this spot, when sprayed with a solution of phloroglucinol in hydrochloric acid, gave an intense red color which is characteristic for wood lignin.

In chromatography with other systems of solvents it was shown that spot 3 is nonhomogeneous. Thus, with ligroin-acetic acid-water (50:25:25) it was separated into two spots, each of which fluoresced with a brown color and gave a colored spot with phloroglucinol. In an analogous way, spot 2 with the solvent system ligroin--acetic acid-water (50:25:25) and ligroin-acetic acid--water-chloroform-methanol (70:25:25:20:10) could be separated into two separate spots which in R_f value and color reactions were identified as vanillin and syringic aldehyde (standards, pure vanillin and the alkaline hydrolyzate of birch lignin).

Thus the results of chromatography show that the benzene extract of the bisulfite fraction contains five aromatic aldehydes, of which two have the characteristic dark brown fluorescence and give a red color with the hydrochloric acid solution of phloroglucinol which is characteristic of wood lignin; the third compound gives a violet spot with phloroglucinol and, finally, the remaining ones are vanillin and syringic aldehyde. The last two aldehydes are present in the mixture in very small amounts.

It is important to mention that in all the chromatograms, in addition, there are always present traces of vanillic and syringic acids, which are easily shown by the diazo reagent. It was observed that in the analysis of freshly prepared samples the spots corresponding to these acids were scarely noticeable, while with long keeping of the solutions they increased considerably in size. Hence we can conclude that when the solutions stand, some of the components of the benzene extract are decomposed, and among the decomposition products are vanillic and syringic acids. Experiments carried out to clarify this question confirmed our assumption. We showed that the compounds which give spots 1 and 3 on the chromatogram are very unstable and are easily decomposed under the action of alkalies, acids, and oxidizing agents. Thus, for example, if the solvent is removed from the benzene solution under a vacuum, the precipitate is treated with aqueous 5% KOH solution, the sample remains at room temperature overnight, and is then acidified and once more treated with organic solvents; then spots 1 and 3 have vanished completely from the chromatogram and in their place appear well-defined spots of syringic and vanillic acids.

Hence, the compounds which correspond to spots 1 and 3 contain guaiacyl and syringic groupings in their nuclei. We have assumed that these compounds are derivatives of the guaiacyl and syringyl series with unsaturated side chains which contain aldehyde or ketone groups in the chain. This assumption depends on the following results, which we have obtained in the chromatography of the benzene extracts: namely, the color reactions of spots 3 and 1 with phloroglucinol hydrochloride resemble the color reactions which this reagent gives with wood and some isolated lignin preparations. According to Freudenberg [8], the color reaction of lignin, the appearance of a red color with phloroglucinol hydrochloride, is given by various methoxy and hydroxybenzene derivatives with an unsaturated side chain. Adler and co-workers [9, 10] and Black and co-workers [11] have shown that the color reaction of lignin with phloroglucinol depends on the presence of a coniferyl or sinapic aldehyde and also their methyl ethers. Also, the compounds which we have investigated easily decompose in an alkaline medium at room temperature. According to the results of Kratzl et al., [12] and Wacek [13] it follows that the ability for easy decomposition under the influence of alkalies is possessed by aromatic compounds which have a conjugated system –C=C-C=O in the chain, and among the products of this decomposition are obtained the corresponding aldehydes and acids. Thus, vanillic and syringic acids can be formed in our experiments as a result of complete oxidation of the side chain to COOH.

Preparative Fractionation of the Hydrolyzate

In order to isolate the dark brown fluorescing compounds from the mixture and obtain a sufficient amount for more accurate analysis, we used the method of partition chromatography on a silica gel column. We used a column of silica gel saturated with strong phosphate buffer; the method of work with this column was described in an earlier paper [1]. We passed the original ether solution, obtained by shaking the hydrolyzate of the chagi pigment complex with ether, directly through the column, since special experiments showed that in work with the silica gel column we could avoid the operation of isolating the carbonyl fraction with sodium bisulfite. When the column was washed with a 1% solution of butanol-chloroform, the aromatic acids and pigment substances remained adsorbed on the column, and the components with the dark brown fluorescence passed into the filtrate which had the greenish yellow color characteristic for these compounds. The solvent was removed from the filtrate in a vacuum and the resulting residue was treated with ligroin at 40-50°. This dissolved about 80-85% of the residue. After the sample had stood for 3-4 hours at room temperature, a yellow precipitate with an oily consistency came down. The clear greenish yellow solution was poured off from the precipitate into a crystallizer and remained overnight at 3-5°. On the next day a precipitate of greenish yellow needle-shaped crystals had separated on the bottom and walls of the crystallizer in compact clumps. The crystals were collected in a yield of about 30% with respect to the weight of the starting residue. The filtrate was evaporated in a vacuum and again placed in a refrigerator. After standing for several days, the filtrate gave a greenish yellow, oily product which we could not obtain in crystalline form. The yield of this product was about 25% of the weight of the original residue.

Study of the crystalline material. The crude crystals were recrystallized from ligroin; they melted at 70-72°. The crystals dissolved easily in ether, chloroform, benzene, alcohol, acetone, ethyl acetate, and acetic acid; they dissolved in water and ligroin with heat.

The solution of the crystals gave a positive reaction for carbonyl groups: reduction of an ammoniacal solution of silver nitrate and Fehling's solution, restoration of the color of the fuchsinsulfurous acid, a red color with sodium nitroprusside in the presence of alkali, and the formation of phenylhydrazones and semicarbazones. With ammonia and alkali metals we obtained a clear orange-yellow salt.

The molecular weight of the crystals was determined according to Rast, by melting with camphor, and by the cryoscopic method. The results of the analysis did not give sufficiently concordant data in different experiments. On the average, the molecular weight of the crystals varied from 300-400.

Determination of the elementary composition gave the following results: C, 54.41%, H, 5.84%; content of methoxyl groups, OCH_3, 25.8%. The crystals in chloroform solution decolorized a bromine solution in the same solvent without evolution of hydrogen bromide, which, as is known, shows the presence in the molecule of unsaturated double bonds.

The substance is very unstable, easily oxidized and decomposed under the influence of alkalies, sulfuric acid, etc. In an alkaline medium the crystals change color at once, and the solution becomes at first red, then dark brown. Among the decomposition products are obtained syringic acid, whose yield, as analysis showed, varied in different experiments from 30-35% of the weight of the crystals. As already mentioned above, the crystalline substance gave an intense red color with phloroglucinol hydrochloride, which, according to the literature data [8-11], indicates the presence of a free coniferyl or sinapic aldehyde grouping in the molecule. The solution of the crystals had the characteristic dark brown fluorescence in the ultraviolet. Thus, from the preceding data it follows that the crystalline substance which we studied belongs to compounds of the syringyl series. This compound has an unsaturated side chain which is easily decomposed in an alkaline medium. It contains an aldehyde group and gives a red reaction

with phloroglucinol hydrochloride, properties of sinapic aldehyde and its methyl ether. It has a higher molecular weight than sinapic aldehyde, is a less methoxylated compound, and differs in its fluorescence (dark brown instead of blue). Recently Hibbert [14] and Kratzl and Schweers [7] have studied the process of ethanolysis of lignin, using a number of monomeric propyl-phenol compounds which are derivatives of the guaiacyl and syringyl series. We can conclude from our analytical results that the crystalline substance which we have isolated belongs to this type of compound.

The presence among the products of hydrolysis of the water-soluble chagi pigment complex of compounds with structures close to that of the unit of lignin, once again confirms our idea that in the synthesis of this complex by the fungus, products of the decomposition of the lignin molecule are used [14]. Manskaya [15] was also inclined to the belief that products of biological decomposition of lignin share in the reactions of secondary synthesis of humus-like compounds which make up the basic organic substances of soil, peat, and coal.

For more complete identification of our crystalline compound we are now carrying out work on the study of its infrared and ultraviolet spectra. We should add that the crystalline substance which we isolated has a high oxidizing potential and is a strong inhibitor: It prevents the fermentation of yeast in concentrations of $14 \cdot 10^{-8}$.

Study of the oily products. During the isolation of the crystalline material, we isolated two products with an oily consistency: a yellow "oil" which precipitated from ligroin at the beginning of the experiment at room temperature, and a greenish yellow "oil" which remained dissolved in ligroin at 3-5°.

From the first oily product we isolated vanillin, syringic aldehyde, and a compound which gave spot 1 in paper chromatography. We did not study the other substance in detail, since it was present in the sample in relatively small amounts. However, it was shown that in an alkaline medium it was decomposed in a manner analogous to our crystals and as a decomposition product syringic acid was also formed. The greenish yellow "oil", as analysis showed, contained compounds with the guaiacyl nucleus which, like the yellow crystals, fluoresced with a brown color, gave an intense reaction with phloroglucinol, and easily decomposed in an alkaline medium. Among the decomposition products of the oily substance we noted vanillic acid, 20% of the initial weight; we have not yet succeeded in isolating crystalline compounds with the guaiacyl nucleus. Work on this oily product is continuing. The results of the present work thus confirm our earlier suggestion [16] that monomolecular structures of lignin units form the basis of the structure of the water-soluble chagi pigment complex.

SUMMARY

In the study of the hydrolysis products of the water-soluble chagi pigment complex we have isolated, in crystalline form, a compound which contains the syringic nucleus with an aliphatic side chain. On the basis of the reactions of this compound it can be considered a derivative of sinapic aldehyde.

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A COMPARISON OF THE ANTIGEN PROPERTIES OF PURE ANTIBODIES AND OF NONSPECIFIC γ-GLOBULINS

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The problem of similarity and difference between individual antibodies, as well as between antibodies and nonspecific γ-globulins, is of considerable interest from the point of view of providing an approach to the understanding of the nature of the specific action of antibodies. It has been shown by a number of investigators that most of the antibodies – in particular, the precipitins of rabbits – do not differ markedly either from each other or from the nonspecific γ-globulins in their electrophoretic mobility, sedimentation rate, amino acid composition, or molecule size, as well as by other properties [1-3]. No differences were found in respect to the antigenic properties of similar antibodies, when various preparations containing antibodies were used as antigens for immunization purposes [4-5]. In the course of immunization of an animal by a preparation containing some kind of antibody obtained from an animal of another species, there are formed antibodies which react equally with the preparations containing the said antibody, as well as with preparations containing all of the other antibodies or nonspecific γ-globulins of the species of animal whose proteins were used for immunization.

However, an essential defect of more recent investigations consists in the fact that various preparations (immune sera, globulin fractions, precipitates) containing a series of other proteins besides the antibodies (instead of the pure antibodies) were used for immunization. It was therefore of importance to study this problem where preparation of pure antibodies were being used, as far as the resulting picture would not be obscured by the presence of large amounts of foreign antigens. Since one of us [6] developed a method permitting the securing of antibodies in pure form, we decided to compare the antigenic properties of separate pure antibodies obtained by this method, as well as to compare them with the antigenic properties of nonspecific γ-globulins.

EXPERIMENTAL

The experiments were conducted on rabbits which were simultaneously immunized by two foreign proteins, horse serum albumin (HoSA) and human serum albumin (HuSA). Each of the antibodies appearing in the rabbit blood was extracted separately by a corresponding absorbent of our method. Hens were immunized by the isolated pure rabbit antibodies (anti-HoSA and anti-HuSA), where each hen received only one of these antibodies. Besides, an additional group of hens was immunized by nonspecific γ-globulins isolated from the blood of a nonimmunized rabbit. After antibodies against the introduced proteins appeared in the hens' blood (e.g., antibodies against the antibodies, or antibodies against non-specific γ-globulins), the hens were fully bled and immunochemical studies performed to study how each of the immune area obtained reacted with each of the originally used antibodies and the nonspecific γ-globulins.

METHODS

The rabbits were immunized by twice-recrystallized horse serum albumin (HoSA) and human serum albumin (HuSA) isolated by means of alcohol.

Securing of immune rabbit sera. Rabbits of 2.0-2.5 kg were immunized by the simultaneous introduction of two foreign proteins (HoSA and HuSA) in increasing amounts (from 1 to 3 mg) on alternate days for a period of three weeks. After an interval of 1-3 months, the animals received 25-50 mg of each of these antigens. After 6-10 days the animals were fully bled, and the obtained serum was kept in a refrigerator.

Isolation of antibodies from the immune rabbit serum. The pure antibodies were isolated from the immune rabbit serum by means of immune-absorbents. These consisted of a cellulose powder on which the protein used for immunization was fixed by means of N-(M-nitrobenzyloxy)-methylpyridine. The serum, which contained two antibodies (anti-HoSA and anti-HuSA), was heated for 30 min at 56° C to inactivate the complement, after which it was passed first through the column with HoSA fixed on cellulose, and subsequently through a column with the fixed HuSA. Here the antibodies joined the corresponding fixed antigens. The nonspecific serum proteins were removed by passage of a 1% solution of sodium chloride (pH 7.0). Subsequently, the antibodies to HoSA and HuSA were eluted from the first and the second immune-absorbents, respectively, by means of a sodium chloride solution of the

same concentration (pH 3.2). The obtained antibodies did not contain admixtures detectable by electrophoretic or sedimentation analysis, and in a number of instances were fully precipitated by the addition of the corresponding antigen. The details of the method were reported earlier [6].

Securing of non-specific γ-globulins. These were isolated from the serum of a nonimmunized rabbit by means of electrophoresis in a starch block. The purity of the obtained protein was verified by paper electrophoresis [7].

Immunization of hens. Ten to twenty mg of a protein under study were introduced once or twice into the central subclavicular vein of 1.5-2.0-kg Leghorn hens. After one or two weeks the hens were fully bled, and the obtained sera were kept in a refrigerator.

Immunochemical analysis of the sera. This was conducted by our modification [8] of the method of Heidelberger [9]. The determination consisted in the precipitation of the antibodies by various amounts of the corresponding proteins, followed by a rinsing of the precipitate from nonspecific proteins and the determination of the amount of protein in the precipitate by Lowry's method [10].

RESULTS AND DISCUSSION

The experiments showed that in all instances of immunization of hens by means of any of the rabbit antibodies, anti-antibodies appeared in their blood, which reacted not only with the particular antibody, but also with other antibodies, as well as with the nonspecific γ-globulins. And, on the other hand, upon the immunization of the birds with nonspecific rabbit γ-globulins, antibodies were invariably formed which reacted not only with the nonspecific γ-globulins, but with the rabbit antibodies as well.

These data are in agreement with the results of a number of earlier investigators [3,5]. The existence of common antigenic properties in the nonspecific γ-globulin and antibodies indicates the presence of identical groupings in the molecules of the proteins under study.

But, contrary to the earlier reported data, we were also able to demonstrate definite differences in the antigenic properties of individual rabbit antibodies. In addition, the difference between the antigens and the nonspecific γ-globulins emerged.

These differences became evident during the drafting of the curves on the relationship between the amount of precipitate and the amount of antigen added to the immune serum. It appeared that such a curve for every individual protein under study did not coincide with that for the other proteins. The curves' falling apart was especially pronounced in the zone of excess antigen.

Figure 1 represents the results of experiment No. 5 on the serum of a hen immunized with rabbit antibodies against horse serum albumin. The serum reacted not only with the homologous antibody, but also with the rabbit antibody against human serum albumin and the nonspecific γ-globulins. At the same time, the curves for each of these proteins differed somewhat from each other, especially in the region of excess antigen.

An analogous picture can be seen in Figures 2 and 3, which represent the results of experiments Nos. 10 and 7 with the sera of hens immunized with rabbit antibody against human serum albumin and nonspecific rabbit γ-globulin, respectively.

We wish to point out that in various sera the ratio curve varied for each of the proteins under study. In some experiments (Figs. 1 and 2) the addition of the protein

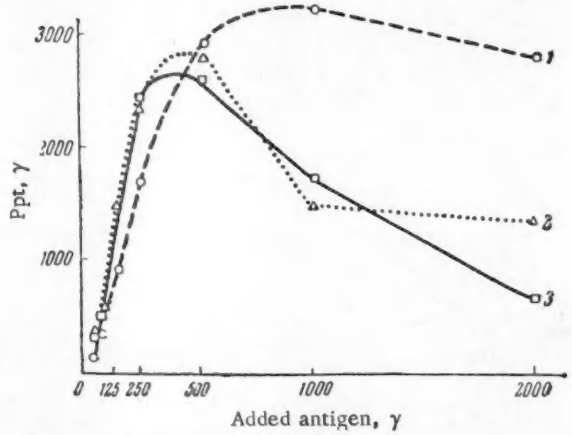

Fig. 1. Relationship between the amount of precipitate and the amount of added antigen in the serum of a hen immunized with rabbit antibodies against horse serum albumin. Added: 1) Rabbit antibody against horse serum albumin; 2) rabbit antibody against human serum albumin; 3) nonspecific rabbit gamma-globulin.

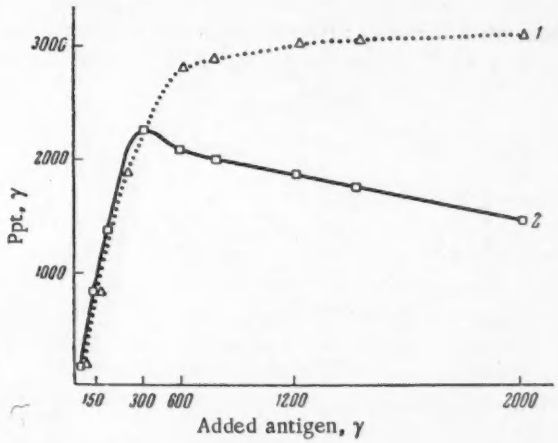

Fig. 2. Relationship between the amount of precipitate and the amount of added antigen in the serum of a hen immunized with rabbit antibodies against human serum albumin. Added: 1) Rabbit antibody against human serum albumin; 2) nonspecific rabbit gamma-globulin.

with which the hen was immunized resulted in larger precipitates. In other cases, however, the reverse relationships took place. Thus, for instance, in experiment No. 6, where the hen was immunized with antibodies against horse serum albumin, an immune serum was obtained which reacted more intensively not with this antibody, but with the nonspecific γ-globulin and with another antibody (Fig. 4).

Besides a marked variation in the reaction of individual hens to immunization with a particular protein, we also observed a certain dependence of the obtained results from the type of antigen used. Similar complicated relationships were observed earlier by Treffers [4] in preparations of nonspecific γ-globulins obtained from sera of horses. Taking this into consideration, it is preferable, in individual problems, to use the same serum and definite type of antigen.

Since each of the immune sera obtained was able to react not only with the antigen but with the nonspecific γ-globulin as well, the question arose of whether it would be possible to obtain an immune serum which would react only with antibodies, by precipitating the antibodies against nonspecific γ-globulins. To obtain such a serum, a preliminary determination was made to ascertain what amount of nonspecific γ-globulins had to be added to obtain a precipitate of maximum size. Then, the required quantity of nonspecific γ-globulin was added to the serum, the precipitate was removed, and a determination of the amount of antibodies in respect to the nonspecific γ-globulin and the antibody under study was made in the supernatant fluid.

Figure 5 describes one of such experiments (Nos. 11 and 12), which was conducted with serum used also in experiment No. 10 (Fig. 2). It can be seen from I of Figure 5 (see the curve in Fig. 2), that the amount of precipitate produced on the addition of antibodies to the serum under study was much larger than the amount of precipitate formed on the addition of the same amount of nonspecific γ-globulin. In order to remove the antibodies against the nonspecific globulin, this very antigen was added to the serum in an amount of 375 γ/ml of serum, since the addition of the said amount yields a maximum precipitate (Fig. 2). The precipitate was separated by centrifuging, and the supernatant fluid was used for the determination of antibodies against nonspecific γ-globulin, as well as against the corresponding rabbit antibody. It was found that as a result of the precipitation of the antibodies against nonspecific γ-globulins, a sharp reduction of the amount of precipitating antibodies against antibodies also took place in the serum (II of Fig. 5). Repeated addition of 375 γ of nonspecific γ-globulins to the already treated serum leads to a further lowering of its anti-antibody content (III, Fig. 5).

It was possible to obtain a better separation of antibodies contained in the serum under study where the amount of antigen used for the precipitation of antibodies against the nonspecific γ-globulins was less (150 γ/ml) than the amount required for a maximum precipitate. Here, it was possible to achieve a somewhat selective precipitation of the antibodies against the nonspecific γ-globulins, while preserving in the serum a large amount of antibodies against antibodies (IV, Fig. 5). While the amount of antibodies against antibodies in the original serum exceeded the amount of antibodies against the nonspecific γ-globulins by 80% it increased to 420% following the reduced precipitation.

One can presumably expect a still more complete separation of the mixture of antigens under study by means of fractionating precipitation with small portions of antigen, thus obtaining an immune serum which will react exclusively with antibodies.

The results obtained indicate the presence of definite antigenic differences between the individual antibodies, as well as between the antibodies and the nonspecific γ-globulins. These data differ from those of some earlier publications. This is evidently due to the fact that in our experiments use was made of pure native antibodies, and not various preparations containing, besides antibodies,

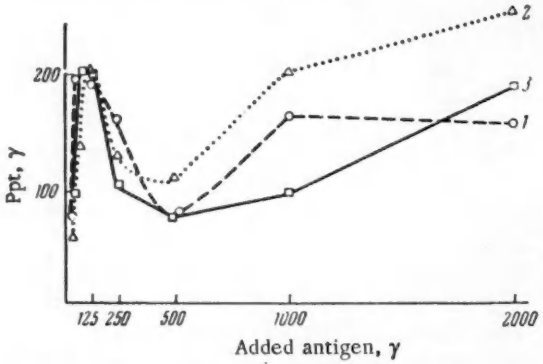

Fig. 3. Relationship between the amount of precipitate and the amount of added antigen in the serum of a hen immunized with nonspecific rabbit gamma-globulins. Additions: as in Fig. 1.

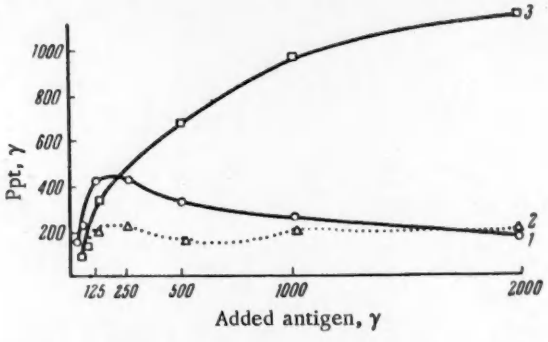

Fig. 4. A more intensive reaction of the serum of a hen immunized with rabbit antigens against human serum albumin, with the nonhomologous, as compared with the homologous antigen. Additions: as in Fig. 1.

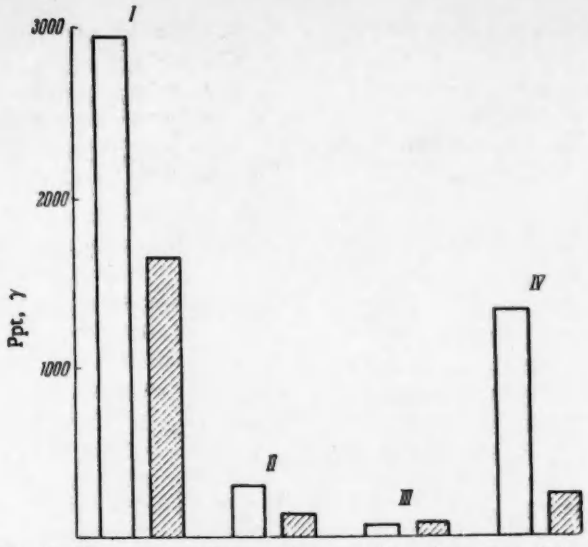

Fig. 5. The amount of precipitate resulting from the addition of antigen (rabbit antibody against human serum albumin) and of nonspecific gamma-globulin to the whole immune serum, and to the same serum after a preliminary precipitation of a portion of the antibodies. I) Untreated serum; II, III, IV) serum from which a portion of antibodies was preliminarily precipitated by the addition of nonspecific globulins in quantities of 375, 750, and 150 micrograms/ml, respectively. The white columns: where an antibody was added; the shaded columns: where nonspecific gamma-globulin was added.

many other proteins. The results reported here are in agreement with the earlier findings by one of us concerning some differences in the electrophoretic mobility of individual antibodies and nonspecific γ-globulins [6], as well as with the data on some degree of independence in the biosynthesis of each of these proteins [11, 12]. Our results are also in good agreement with the investigations which appeared in print during the preparation of our paper [13], where the difference in the antigenic properties of antibodies and nonspecific γ-globulins is shown by means of other methods (sensitization and desensitization).

We did not succeed in obtaining an immune serum which would react with antibodies and would be absolutely inert to nonspecific γ-globulins, but definite progress in this direction has already been made. Further work in this direction is required; use should be made of the newest immunochemical methods.

SUMMARY

When hens are immunized with pure antibodies isolated from rabbit serum, antibodies appear in their blood which react not only with the antibody used in their immunization, but also with the other rabbit antibody, as well as with rabbit γ-globulin. On the other hand, upon the immunization of hens with nonspecific rabbit γ-globulins, antibodies are formed which react not only with the nonspecific rabbit γ-globulins, but with rabbit antibodies as well. However, each of the antibodies and the nonspecific rabbit γ-globulins react in a nonidentical way with each of the immune sera obtained. These differences are most clearly pronounced between the individual antibodies, as well as the antibodies and the nonspecific γ-globulins, becoming evident in the zone of excess of antigen. These differences are observed in all cases, while there is a variation in various sera in the rate of the course of the curves for each of the proteins under study.

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THE EFFECT OF HIGH-ENERGY N-PHOSPHORYL COMPOUNDS ON THE CONTRACTILE ACTIVITY OF GLYCERINATED MUSCLE FIBERS

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Goodall (1956) described the relaxing effect of phosphorylcarnosine on glycerinated muscle fibers [1]. The addition of phosphorylcarnosine to the medium in which the fibers were suspended elicited a response similar to that produced by phosphorylcreatine [2], namely, the relaxation of the glycerinated muscle fiber when the pH of the medium was adjusted from pH 7.0 to 6.0.

Phosphorylcreatine is the natural relaxing factor in muscle, since it participated in the rephosphorylation of ADP [3]. However, phosphoryl-group transfer from phosphorylcreatine is not the only means of ADP resynthesis, since this can also be accomplished by the action of myokinase on ADP [4], as well as by the latter reacting with phosphoenolpyruvate [5]. Accordingly, myokinase and phosphoenolpyruvate can also be regraded as relaxing factors for muscle. It was therefore proposed by Goodall that phosphorylcarnosine was concerned in phosphorylation of ADP and thus participated in muscle metabolism.

Experiments in vitro failed to demonstrate any phosphoryl group transfer from phosphorylcarnosine to ADP [6]. No demonstrable improvement in the work of a neuromuscular preparation could be demonstrated when phosphorylcarnosine was added to the surrounding medium [7]. All attempts to demonstrate the presence of phosphorylcarnosine in muscle were unsuccessful. Furthermore, the results of Cain, Delluva and Davies [8] definitely excluded the possibility of the participation of phosphorylcarnosine in muscle metabolism.

These results suggested to us that, contrary to Goodall's assumption, phosphorylcarnosine is not specifically a relaxing principle for muscle, and that other high-energy N-phosphoryl derivatives, similar to phosphorylcarnosine, would have a relaxing effect on muscle. This possibility was checked by testing a number of phosphorylated peptides and amino acids as relaxing factors for a glycerinated muscle fiber preparation.

METHODS

The assay method used in the present work was that of Goodall and Szent-Gyorgyi [2], in which repeated contraction-relaxation cycles are obtained with a glycerinated muscle preparation in the presence of phosphorylcreatine. The compounds tested for relaxing factor activity on the glycerinated muscle fiber, under conditions of isometric contraction, were the N-phosphoryl derivatives of creatine, carnosine, anserine, β-alanine, histidine, lysine and norleucine.

The N-phosphoryl derivatives were prepared by a method previously described [9]. The preparations were analyzed for labile phosphorus (P_{lab}) according to Fiske and SubbaRow after the removal of $P_{inorganic}$ by precipitation with magnesia mixture. In addition, the phosphoryllysine preparation was also analyzed for nitrogen (total N) after wet-ashing, using the procedure of Conway. Phosphorylcarnosine preparations were also checked by colorimetric estimation with the diazo reaction after 10-min hydrolysis in 1 N HCl on the boiling water bath. A comparison of the values for P_{lab} with the results of the diazo reaction before and after hydrolysis allowed to calculate the ratio of mono-and di-phosphorylcarnosine in the preparation [9].

The preparation of glycerinated fibers. Long, thin, light psoas muscle of freshly killed rabbits were placed into Koch vessels containing 50% aqueous glycerol, cooled on ice. After one hour the coarse bundles were divided into finer ones, not exceeding 0.5–1.0 mm in diameter. The fibers were extracted in 200 ml of 50% glycerol for two days at 0°, transferred into fresh 50% glycerol containing 0.05 N $NaHCO_3$, and the extraction was continued at -18 to -20°. The fibers were stored in this solution for prolonged periods (5–6 months). They could be used as soon as after 5 days' storage at - 20°. According to Goodall and Szent-Gyorgyi [2], at this stage the muscle fibers are completely devoid of relaxing factor.

The selected fibers were kept at room temperature in 20% glycerol for at least one hour prior to the experiment.

The preparation of muscle extracts. Minced rabbit dorsal muscle was extracted for one hour at 0° with an equal volume of 0.05 N $NaHCO_3$. After centrifuging, the extract was dialyzed 24 hours in cellophane bags against running tap water. The dialyzed extract was

* The participation of G.A. Solov'ev in this work is acknowledged.

cleared by centrifugation and stored at 0°. Fresh extracts, and those stored for up to three weeks, were used in the present work.

Recording instrumentation. The apparatus for recording the isometric contraction and relaxation of the glycerinated muscle fibers consisted of three components. The first component comprised a small glass vessel, made from a section of tubing 1.3 cm in diameter and 2.5 cm long, rigidly fastened to a Petri dish. The bottom of the vessel was closed by a bark cork into which a small silver hook was fitted. The bottom of the vessel was covered with a layer of pure paraffin wax. The second component of the apparatus was an isometric lever, prepared from a thin glass rod, 0.5 mm in diameter and 10 cm long. One end of this rod was fixed by means of a cork to a stand, while the other was suspended freely over the vessel. The very slight oscillations of this free end were recorded by the third component – a type MBS-1 microscope. One of the eyepieces of the microscope carried a micrometer scale, and the magnification was arranged so that one division of the scale corresponded to a movement of the free lever end of one mm.

Preparation of solutions. The solutions were set up basically according to Lorand [3]. The medium (for each filling of a vessel) consisted of: 0.13 ml of 0.024 M $MgCl_2$; 0.13 ml of 2.4 M KCl, 0.5 ml of 0.04 M ATP; the N-phosphoryl compound tested in amounts described below; 1.0 ml of the dialyzed muscle extract; water – to 4.0 ml.

Two parallel samples were prepared, which contained Mg^{++}, K^+, ATP, muscle extract, and one of the phosphorylated compounds. At the same time, two other samples were prepared which contained only Mg^{++}, K^+, ATP, and muscle extract. The behavior of the muscle fibers in the latter two samples served as a control for the characteristics of the reaction of muscle fibers in the presence of phosphorus derivatives in the solution. Finally, two more samples were prepared which contained Mg^{++}, K^+, ATP, muscle extract, and phosphocreatine in amounts corresponding to 10 m moles P_{lab}.

In all three cases the solutions were set up in duplicate. One of the solutions in each pair was adjusted to pH 7.2 by the addition of 0.1 N NaOH, while the other was brought to pH 6.2 by passing CO_2 through it. A drop of octyl alcohol was added to the solutions to avoid frothing. The pH of the solutions was first measured potentiometrically, and later using universal indicator externally.

Prepared muscle fibers were checked microscopically for possible damage, selected, fixed in the vessel and tied with a fine silk thread to the free end of the lever.

Experimental procedure. The experiment was begun by adding the solution at pH 7.2 to the vessel; the deflection of the lever was measured with the microscope and noted. After one minute the pH 7.2 solution was removed was removed and the vessel charged with the pH 6.2 solution. A reading of the deflection of the lever was again taken. After one minute the pH 7.2 solution was again substituted for the pH 6.2 medium, followed after one minute by the latter, etc. The alternate changes in pH caused repeated contraction-relaxation cycles. Seven readings were taken for each medium change: at the commencement of the addition of the medium, at the completion of the addition, at 15, 30, 45 and 60 sec and, finally, after the removal of the medium. Fourteen points were thus available for each complete contraction – relaxation cycle. Each medium change, from the commencement of emptying to the complete filling of the vessel, occupied approximately 30 sec. When plotted, the points produced smooth curves for the cycles. The lever deflection (in mm plotted against time (in min).

As a rule, basal (background) activity of the fibers was checked at the commencement of each experiment by allowing the preparation to go through two or three cycles with a control solution. The effect of the phosphoryl derivative under investigation was checked immediately afterwards (two to three cycles) and, finally, the same preparation was allowed to go through several cycles with solutions containing phosphorylcreatine. This procedure allowed the comparison of the compound in question with phosphorylcreatine as a relaxing factor, at the same time affording information of background activity.

RESULTS AND DISCUSSION

The effect of phosphorylcreatine. The curves obtained by plotting the background activity and the activity in the presence of phosphorylcreatine were used as a criterion of the condition and suitability of any preparation. The background curve represents the behavior of the glycerinated muscle fiber in solutions with alternating pH, containing no high-energy compounds other that ATP. Alteration of medium pH can be shown (cf. Fig.) to cause slight oscillation of the lever. This is illustrated in the first portions of the curves (part I) shown on the figure (left-hand portions of curves a, b, c, d, e, and f).

The effect of phosphorylcreatine on the glycerinated muscle fiber preparation was originally demonstrated by Goodall and Szent-Gyorgyi [2]. This observation was repeated many times in this laboratory. Thus, on all the plots shown, (figure) the characteristic rises and falls (contractions and relaxation, respectively) can be clearly seen in response to pH alteration between 6.2 and 7.2 in the presence of 10 m moles of phosphorylcreatine (right-hand portion III of each curve).

The effect of phosphorylcarnosine. The preparations of phosphorylcarnosine employed in the present work were mixtures of the mono-and di-phosphoryl derivatives.

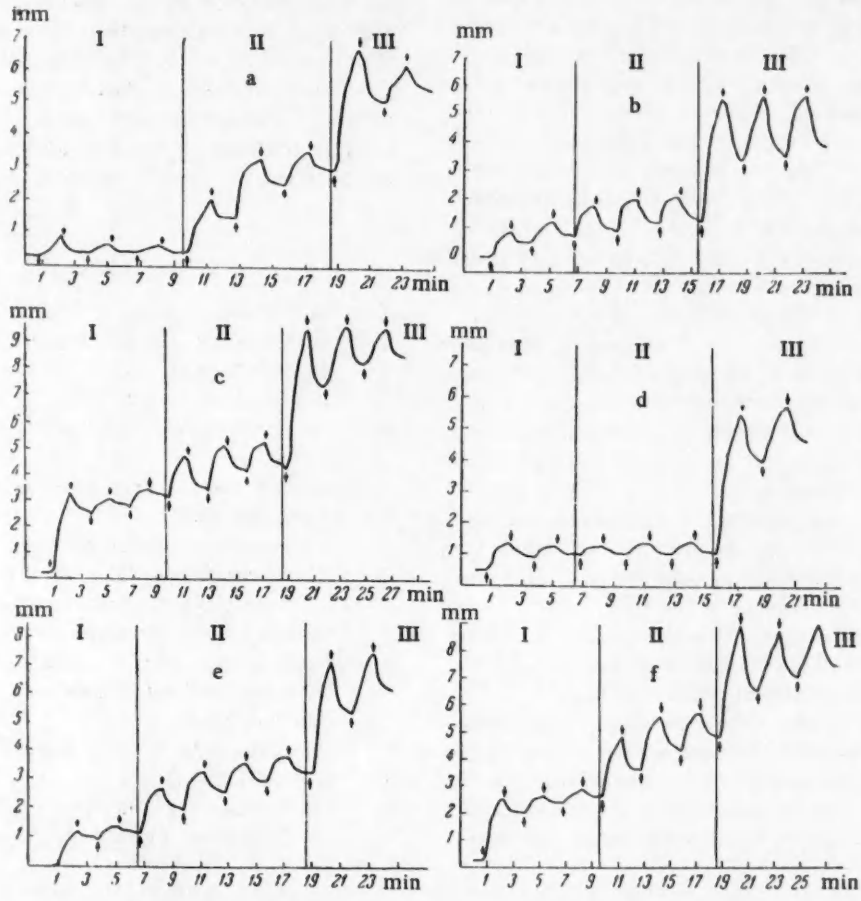
The effect of phosphorylcarnosine on the glycerinated muscle fiber was first demonstrated by Goodall. The observation was confirmed by the present work.

However, in the present experiments phosphorylcarnosine was tested in three different concentrations – in solutions containing 10, 20 and 30 m moles of P_{lab}, respectively – since the possibility was expressed that only one phosphoryl group of phosphorylcarnosine, namely that associated with the β-alanine moiety, was responsible for the effect on the muscle fiber (curve a, figure).

The results of the experiments with phosphorylcarnosine suggest that the effect of this compound is similar to that of phosphorylcreatine. It was also observed that the increase in phosphorylcarnosine concentration in the medium caused a greater response of the muscle fiber in the form of increased amplitudes of contraction and relaxation. However, the absolute effect of phosphorylcarnosine was smaller than that of phosphorylcreatine, even in experiments when the former was present in higher concentrations (in terms of P_{lab}) than the latter. The rather pronouced effect of phosphorylcreatine was expressed in the amplitude of the contraction elicited, apart from that of the relaxations. As seen from the figure (portion III), all curves showed considerable rise at the point where fibers were immersed in a solution containing phosphorylcreatine. The relaxation, which followed these initial contractions upon change of medium pH, was never as large as the contraction, the relaxation thus being incomplete.

The effect of phosphorylanserine. The phosphorylation of anserine under the conditions employed brought about the formation of a monophosphoryl derivative, the phosphoryl group being located on the nitrogen of the β-alanine amino group [10].

As in the case of phosphorylcreatine, the amount of the derivative added was equivalent to 10 m moles of P_{lab}. The results obtained (curve b, figure) were similar to those in the presence of phosphorylcarnosine, that is, the contraction–relaxation cycles had an amplitude



The effect of N-phosphoryl derivatives on the contractile activity of glycerinated muscle fibers. I) Effect of control solutions (background activity); II) effect of solutions containing the respective N-phosphoryl amino compounds: a) Phosphorylcarnosine; b) phosphorylanserine; c) phosphoryl-β-alanine; d) phosphorylhistidine; e) phosphoryllysine; f) phosphorylnorleucine. III) Solutions containing phosphorylcreatine. Arrows pointing downwards indicate the point of addition of pH 7.2 medium; those pointing upwards – the addition of pH 6.2 medium.

above the background activity, but not as large as that of phosphorylcreatine responses.

The effect of phosphoryl-β-alanine and phosphorylhistidine. The effect of the phosphoryl derivatives of these two amino acids was of particular interest since they are the two components of the peptide carnosine.

The alternate immersion of the muscle preparation in solutions of pH 7.2 and 6.2, respectively, containing phosphoryl-β-alanine (equivalent of 10 m moles P_{lab}) produced contraction and relaxation similar in amplitude to those produced by phosphorylcarnosine and phosphorylanserine (curve c, figure).

In contrast to the other compounds tested so far, phosphorylhistidine was without effect on the glycerinated muscle fiber preparation (curve d, figure); the curves representing activity in the presence of this derivative did not differ from those of background activity. It is possible that this lack of effect is due to the different chemistry of phosphorylhistidine, where the phosphoryl group is bound to a ring nitrogen of the imidazole, while in all the other compounds it is bound to an aliphatic nitrogen (N-phosphoryl derivatives of α-amino compounds are known to be highly unstable). This theory gains support from experiments with phosphorylcarnosine, where a greater effect was observed when the muscle was immersed in a solution containing the preparation in amounts equivalent to 10 m moles P_{lab}, than was the case when phosphorylcarnosine containing 10 m moles of total P was added.

The effect of phosphoryllysine and phosphorylnorleucine. In order to check the validity of the assumption that phosphorylcarnosine was a specific relaxing factor (cf. Goodall), the muscle preparation was checked for response in the presence of the phosphoryl derivatives of lysine and norleucine, two amino acids which are not constituents of carnosine.

The norleucine derivative was a monophosphoryl compound, while the lysine derivative was a diphosphoryl, both the amino groups having been phosphorylated (as shown by P and N analysis).

Both compounds tested—phosphoryllysine in concentrations corresponding to 10 and 20 m moles P_{lab} per solution and phosphorylnorleucine equivalent to 10 m moles P_{lab}— produced the same result, the response being well above the background activity levels, but below that of phosphorylcreatine (curves e and f, figure).

It is of interest to note that none of the compounds tested produced muscle relaxation to initial, pre-contraction levels. This was particularly pronounced in the case of phosphorylcreatine. The impression gained with phosphorylanserine was that here the relative relaxation was more complete.

The very pronounced relaxing effect of phosphorylcreatine, and the weaker and comparable effects of the other phosphoryl derivatives (with the exception of phosphorylhistidine), suggest that phosphorylcarnosine cannot be considered a specific relaxing factor for the glycerinated muscle fiber.

SUMMARY

The effect of phosphorylcreatine, and that of phosphoryl derivatives of carnosine, anserine, β-alanine, hystidine, lysine and norleucine on the contraction and relaxation of glycerinated muscle fiber preparations was tested under conditions of isometric contractions caused by fluctuations of medium pH.

Phosphorylcreatine was shown to produce the most pronounced contraction and relaxation response. With the exception of phosphorylhistidine, all the other phosphorylated compounds produced approximately similar responses, considerably lower than those produced by phosphorylcreatine. It was concluded that phosphorylcarnosine was not a specific relaxing factor for muscle.

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RADIATION INACTIVATION OF CELL NUCLEI AS THE METHOD OF STUDY OF THEIR ROLE IN THE DEVELOPMENT OF RESPIRATION IN FISH EMBRYOS

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Changes in the rate of respiration in the process of growth are at the present time the subject of much study. Though the curves obtained for different forms sometimes differ from each other, in all cases, however, the amount of oxygen required by the embryo per unit time increases many times during the course of development. This cannot simply be explained as an increased energy requirement brought about by an intensification of the use of the respiratory apparatus already existing in a prepared state in the unfertilized egg. It is therefore possible to refer to the development of the respiratory apparatus, which is closely reflected in the rate of respiration.

The increased oxygen demand seems to be clearly a secondary mechanism, bringing about the normal path of morphogenesis and activity of the embryo. At the same time it indicates some independent process, which can serve as a characteristic of development, like any other morphological or functional feature. The development of the intracellular respiratory apparatus consists not only in an increased concentration of respiratory enzymes, but includes also the formation of morphological structures, in which the enzymes are localized. The main location of the respiratory enzymes seems to be the mitochondria.

The development of the respiratory apparatus can serve as a satisfactory object for the study of the mechanisms of ontogeny, since it is composed of a similar series of successive synthetic processes as are characteristic for any morphogenetic process. Besides this, insofar as the development of respiratory apparatus is delimited by the formation of only intracellular structures, it can serve as a model for the understanding of the processes of development, preceding and predetermining the occurrence of more complex cellular interactions in the differentiation of tissues and organs.

The investigation of the function of the nucleus in development seems to be one of the central general problems of biology, since it leads to the solution of such questions as the translation of hereditary genotypic information into phenotypic characteristics, and an understanding of the differentiation of cells and tissues when cells divide into equal parts [1]. Some progress has certainly been made up to this time in this important field, since experimental methods of investigation have been devised in classical work for the transfer of nuclei from young embryos to the egg [2, 3], the observation of the fate of the embryo with different combinations of male and female nuclei [4], and in part for the immunological examination of the hybrids [5, 6].

However, very recently, when it was shown clearly in a series of radiobiological studies that the destruction of the embryo by radiation was brought about by irreversible damage to the nuclei [7, 8], it became evident that ionizing radiation could be used as a tool for the investigation of the function of the nucleus in development.

This method was applied by the authors of the present work successfully to young developing fish [9], and is here extended to other objects. The method consists in determining experimentally the range of radiation dosage in which the embryo nuclei are almost entirely inactivated while the cytoplasm remains essentially undamaged. The effect of such doses on the embryo is that some time after the inactivation of the nuclei, development continues, but then stops, and within a short time the embryo dies. It is clear that the development that occurs after the inactivation of the nuclei does so under the control of the cytoplasm, and indicates to what extent, at any given stage of development, the functions of the cytoplasm are determined by the previous activity of the nucleus. The comparison between the development of the embryo, irradiated at succeeding stages, can show at what periods the nuclei

*Here and below we will call the "respiratory apparatus" all the structural complexes and compounds (such as mitochondria and respiratory enzymes) bringing about the consumption of oxygen in the cell.

function and what resulting processes are determined by this function.

METHODS

Ripe sex cells of the groundling Misgurnus fossilis were obtained after making a stimulatory injection of the chorionic gonadotropic hormone (200 international units). Artificial fertilization made it possible to get a large number of synchronously developing embryos. The development up to and during the experiment occured at a temperature of 21.5°C. The stage of development was characterized by the number of hours of normal development that had elapsed. Thus, early blastula occurs at 4-6 hours, the beginning of gastrulation at 10 hours, the end of gastrulation at 17 hours, etc.

Oxygen consumption was determined in a Warburg apparatus, using 20-25-ml flasks. In each flask were put 200-800 fertilized eggs, mixed with 5 ml of water. In this range, as was shown by special experiments, the rate of respiration of one egg was not affected by the amount of eggs in the flask. In order to eliminate bacterial growth, sulfonamide (0.1 mg/ml) and penicillin (100 units/ml) were added to the boiled air-saturated water. The flasks were shaken at 70 oscillations a minute. The experiment was continued for 8-20 hours. The air in the flasks was changed 1-2 times during the course of the experiment to prevent the partial pressure of oxygen falling below 10%. A morphological control showed that under these conditions development was completely normal. Manometer readings were taken every 30 or 60 minutes. The rate of respiration was expressed as microliters O_2 consumed by 100 embryos per hour. This was related to the stage of development existing at the time midway between two consecutive readings.

The irradiation was carried out with a RUP-1 x-ray apparatus. Irradiation treatment: 185 kv, 15 ma without a filter, giving 4000 r/min. The usual dose of irradiation was 40,000 r.

Part of the irradiated spawn was kept as a control in Petri dishes. A morphological comparison of the irradiated spawn in the Warburg flasks and in the Petri dishes showed that the rate and type of development and also the time of death of the embryos after irradiation were identical under these conditions.

EXPERIMENTAL RESULTS

Normal Respiration.

The respiration of one part of the spawn from the beginning of development to hatching (50 hrs) is presented in Figure 1B. It may be seen that during the time of embryonic development the rate of respiration increases approximately 7-8 times. The increase in the rate of respiration is a steady one, and the curve depicting the rate of respiration is almost a straight line. Within the limits of error of the experiment, no significant regular fluctuations in the rate of respiration were observed.

The respiration of young embryos, on which all further experiments were done, is very interesting. In Figures 1A and 2 it may be seen that the increase in the rate of respiration does not start with the beginning of development, but only after 5-7 hours. The subsequent increase in the rate of respiration is steady. This regularity is also found in the respiration of other bone fish [10], in which, in contrast to other experimental objects such as, for example, amphibian embryos [11, 12] or

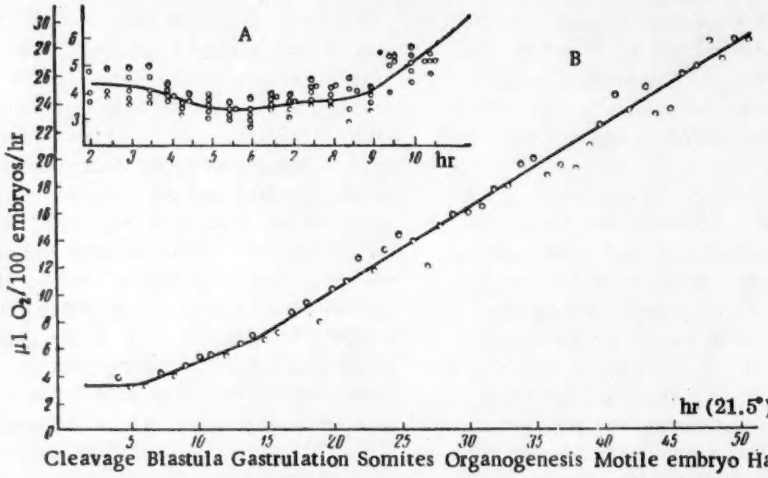

Fig. 1. Rate of respiration during the process of embryonic development in the groundling. Each point represents the mean of results from three flasks. In the left-hand corner is shown the early period of growth; here each point represents the results from one flask.

echinoedia [13], the rate of respiration remains constant up to the time of early-middle blastula, which is the time when, in our experiments [9], the cell nuclei begin to exert their control over development. At this time the rate of respiration suddenly begins its steady increase.

In Figure 2 are also presented the results on the respiration of unfertilized eggs. Here the ripe eggs from the same female as in the control were suspended in water, but no sperm were added. A comparison of the respiration of these eggs with that of the fertilized eggs shows that, during the first hours, the rate of respiration of the unfertilized was the same as that of the fertilized eggs. The difference appeared only at the seventh to eighth hour of development, the increase in the rate of respiration being absent, of course, in the unfertilized eggs. Within a short time some of the eggs begin to die, and the consumption of oxygen in any one flask falls. A careful comparison of the number of dead eggs and the fall in the rate of respiration shows that the death of some of the eggs is the sole cause for the decrease in the oxygen consumption. Right until the moment of death, the rate of respiration of the unfertilized eggs remains constant.

Respiration of Irradiated Embryos

In Figure 3 are presented the results of an experiment in which the sex cells of the groundling were irradiated during fertilization.

As has been established in particular cases [14, 15], and with the present experimental objects [16], the development of embryos after one of the sex cells has been heavily irradiated proceeds almost normally in the early stages of embryogenesis, although in haploid fashion. Lagging of development and malformation appears only in relatively late stages. Many embryos survive until the end of the embryonic period, and even form abnormal swimming larvae. However, irradiation of both sex cells results in the same effect as irradiation of the fertilized egg, that is, development only until late blastula, followed by a cessation of development and then death.

In Figure 3, results of an experiment are presented where the respiration of eggs obtained from one pair of fish was modified. Four possible combinations were investigated:

I. ♂ and ♀ unirradiated (normal development, control); II. ♂ irradiated, ♀ unirradiated (earlier development normal, due to the haploid female nucleus, and the cytoplasm, which was not irradiated); III. ♂ unirradiated, ♀ irradiated (earlier development normal, due to the haploid male nucleus, the cytoplasm being irradiated); IV. ♂ and ♀ irradiated (with the nuclei inactivated, development proceeds only to the late blastula stage).

It is clear from Figure 3 that in early development, up to the seventh hour, the respiration rate of all four variants is the same. However, at a later time, the controls and haploid embryos, the latter with either irradiated or unirradiated cytoplasm, exhibit an increase in the rate of respiration in the usual way. At the same time, the respiration of the embryos with inactivated nuclei remains at the same level as at the beginning of development until the time of death. The results of this experiment clearly show that the increase in respiration during development and the prevention of this increase by irradiation is closely connected with the nucleus, and not with the cytoplasm. In the irradiation of young embryos we are dealing with this process: Radiation does not destroy the respiratory apparatus directly, but only suppresses its development, since it brings about this effect by destroying the nucleus.

In Figure 2 are presented the results of irradiating embryos immediately after fertilization. The results do not differ from those when both sex cells are irradiated.

In Figure 4 is presented the respiration of normal embryos, and of embryos irradiated at various stages of development. It is clear that the effect of doses which inactivated the nucleus (40,000 r) was the same in all the stages of development that were examined: After irradiation at this or some other time, some increase in the rate of respiration continues (as will be shown below, after irradiation in certain stages, this rise is almost absent), then the rate remains constant right up to the time of death of the embryo.

A more exact determination of the level at which the rate of respiration remains constant may be obtained by calculating the mean of a series of readings. An arithmetic mean was calculated from the points lying on the plateau, in the time between the end of the rise in respiration after irradiation and the onset of death of the irradiated embryos. The determination of the onset

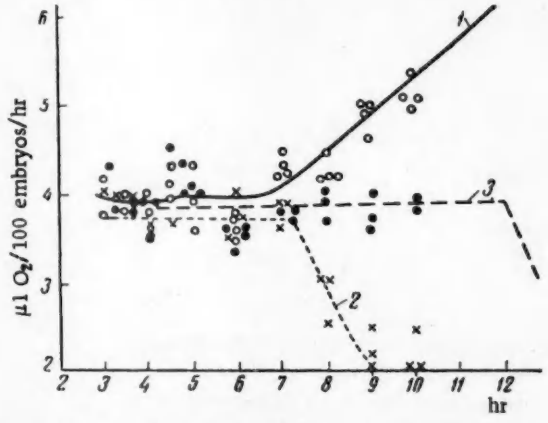

Fig. 2. Rate of respiration of normal and irradiated embryos, and of the unfertilized eggs. Only fertilized eggs were irradiated. Each point represents the results from one flask. 1) control; 2) unfertilized eggs) 3) irradiated embryos

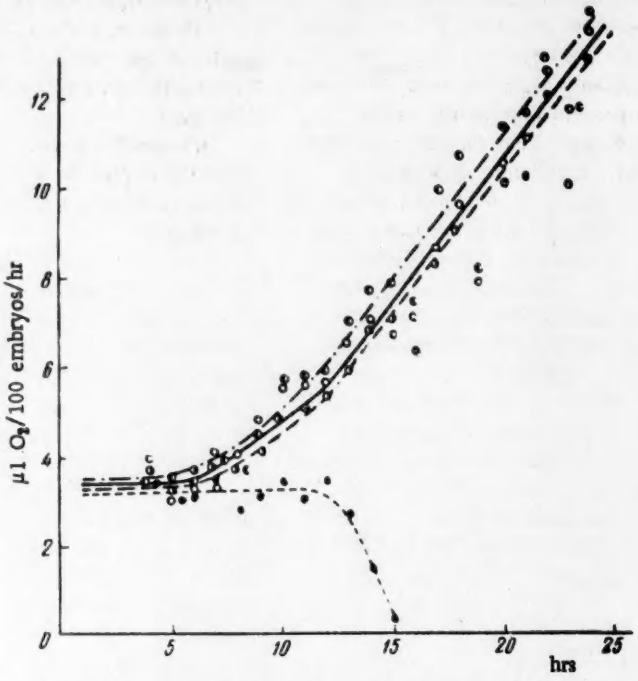

Fig. 3. Rate of respiration of normal embryos and of embryos obtained after irradiation of male, female, or of both gametes. Each point represents the mean of results from two to three flasks. White circles - control; shaded on the left-irradiated sperm; shaded on the right - irradiated egg cells; black circles - both gametes irradiated.

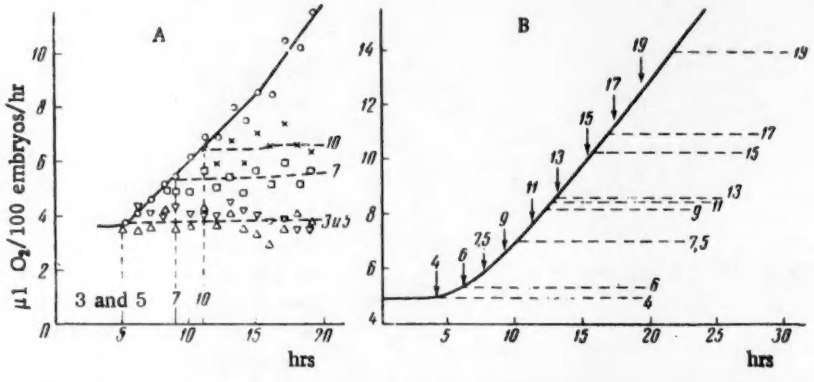

Fig. 4. Rate of respiration of embryos irradiated at various stages of development. Figures denote hours of development at the time of irradiation. A: dotted lines (horizontal) - mean rate of respiration attained after irradiation at a given stage; dotted lines (vertical) - projections of the rates of respiration at the corresponding stage of development. B: points are not shown, but only the mean rates of respiration(see text). Arrows - time at which irradiated.

of death may be made visually and is not difficult; the determination of the end of the rise can be estimated from the graph with some error. However, the wrong inclusion or exclusion of one or two low points cannot result in a large error, since they would have little effect on the magnitude of a mean compounded from 8-15 points.

Dependence of the Cessation of the Rise in Respiration on the Stage of Development at the Time of Irradiation

The level at which the increase in the rate of respiration ceases can be expressed not only as the amount of O_2 consumed per unit time, but also as the stage of development at which this level of respiration normally exists. As may be seen from Figure 4A, a perpendicular can be drawn to the abscissa from a point intersecting it on the horizontal line, corresponding to the level found in the curve of normal respiration. Thus, Figure 4A shows that when eggs are irradiated at either the 3- or the 5-hr stage, the rate of respiration does not increase at all, and remains at the level of the 5-hr stage. When the 7-hr stage is irradiated, respiration reaches a level corresponding to the 9-hr stage, and when the 10-hr stage is irradiated, respiration reaches the level corresponding to the 11-hr stage. Thus, the ability of the respiratory apparatus to develop without nuclear control can be expressed as those stages of development which it can reach after irradiation.

In Figure 5 the summarized results are presented of all the experiments carried out, including those represented in Figure 4.

So that the results obtained could be compared with those of preceding work, the same principle was used in constructing this graph as before [9]. On the lower abscissa are the stages of normal development (in hours) to which the level of respiration reached by the embryos up to this time correspond. On the ordinate are the hours of normal development at the time of irradiation. Each point (points which resulted from different experiments are marked differently) corresponds to the cessation of the rise in the rate of respiration (represented as a stage of development) when irradiated at a particular time of development. The time of irradiation is also depicted on the graph. The straight line joining them is the "times of irradiation". The horizontal distance from each point on this line to the experimentally determined points corresponds to the degree of development which the respiratory apparatus underwent after irradiation, that is, without nuclear control.

Even though in Figure 5 material has been used from a whole series of experiments, which were carried out during the course of several months, even though the

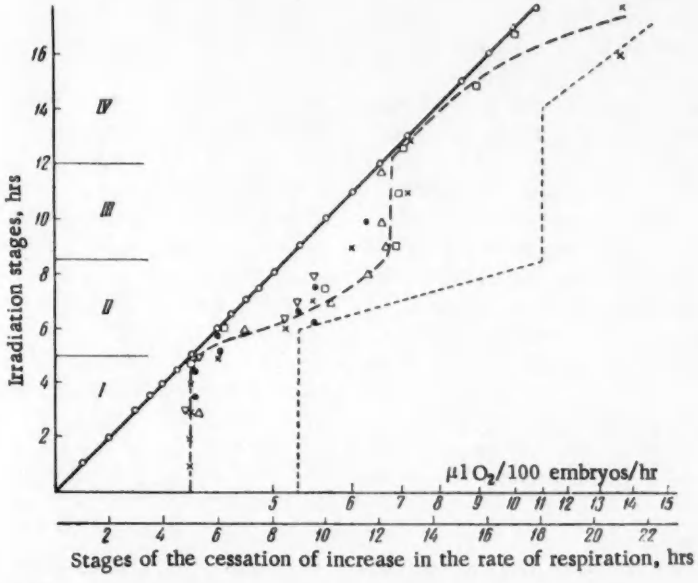

Fig. 5. Dependence of the stage of development, corresponding to the level of respiration attained after irradiation, on the stage of development at the time of irradiation. Compounded results from five experiments. Each point represents one series of the experiment. Solid line - times of irradiation; broken line - cessation of development of the respiratory apparatus; dotted line - cessation of morphological development. For further details, see text and [9].

experiments were not connected and were conducted on material obtained from various sources, and even though there were errors in measurement while the level of respiration was relatively exactly expressed as the stage of development reached, yet the points, when assembled, give a sufficiently regular pattern. The broken line which joins these points is the "cessation of development of the respiratory apparatus". The further it deviates from the line of "times of irradiation", the greater the capacity of the respiratory apparatus to develop without the control of the nucleus beyond the stage at the time of irradiation. Conversely, at those points where the two lines almost meet (5- and 13-hr stages) the development of the respiratory apparatus stops immediately after the inactivation of the nucleus.

In Figure 5 also a dotted line indicated the "cessation of morphological development", which is derived from previous work where the cessation of development was determined on morphological criteria.

As in previous work, the experimental curve may be divided into four parts, which are indicated on the ordinate: I up to 5 hrs, II from 5 to 8 hrs, III from 8 to 12 hrs, and IV from 12 hrs up. The first and, in marked degree, the third periods are characterized by being independent of the time of irradiation (within the same period). During the first period, respiration remains constant over the whole time regardless of whether the nuclei were inactivated by radiation or not. It is obvious that in this period the nucleus does not yet control the respiratory apparatus, since there is no rise in respiration or, consequently, any development of the respiratory apparatus during this period. In the third period also, nuclear control is almost or entirely lacking. Whether the nuclei were inactivated at the beginning or at the end of this period, the respiratory apparatus developed to the level corresponding to an 11-12-hr consumption of oxygen in normal development (early gastrula stage). It is clear that the cytoplasm of the embryonic cells in this period has already attained sufficient "independence" to raise the rate of respiration to the level observed.

Conversely, the second and fourth periods are characterized by a considerable dependence on the time of irradiation: the later within one of these periods the nuclei were inactivated, the higher the rate of respiration reached by the embryo. An irradiation, given 30 minutes later than a previous one, allows a development of the respiratory apparatus corresponding to a 1-1.5-hr later stage. It follows that, during this 30 minutes, the nucleus accomplished some function which was essential to permit these additional hours of development. Clearly, the second and fourth periods correspond to a time of intense nuclear activity in connection with their control of the development of the respiratory apparatus.

In the course of the second period (from early to middle blastula), the rise in the rate of respiration from the middle blastula to the beginning of gastrulation is already being prepared. In the fourth period (from the beginning of gastrulation), the rise in the rate of respiration at the time of gastrulation is prepared.

DISCUSSION

The role of the nucleus in the suppression of respiration by irradiation. From the experiments presented above, it is clear that even very high doses of radiation do not bring about a decrease in respiration directly. This fact has been mentioned repeatedly in the literature [17, 18]. However, the characteristic increase in the rate of respiration during development is quickly suppressed by ionizing radiation. Consequently, one of the reactions in the process of the development of the respiratory apparatus seems to be an object of the destructive effects of radiation.

It is important in all the present work to discover whether radiation directly suppresses the processes of synthesis of respiratory enzymes and formation of mitochondria themselves, these processes occurring apparently in the cytoplasm, or whether it interferes with some initial process in the development of the respiratory apparatus, which takes place in the nucleus. The experiment in which it was found that haploid embryos with intact male nuclei and irradiated cytoplasm exhibited a rate of respiration almost the same as that of controls clearly showed that, under known radiation doses, those processes which are localized in the cytoplasm can function almost normally. But radiation inactivation of the nucleus completely suppresses the rise in respiration.

It is obvious from the results of the present experiments that in the stages examined, the nucleus does not control the process of respiration itself, but appears to be necessary for the development of the intracellular respiratory apparatus.

Periods when the nucleus has a controlling function. From the graph in Figure 5, in which the results of all the experiments are summarized, it is possible to establish not only a relationship between the stage at the time of irradiation and the stage which the development of the respiratory apparatus reached in the cells of the irradiated embryo. The results presented also allow one to judge when the nucleus exerts control over the development of respiration, and what stages of its development are controlled by the nucleus.

Actually, in the first period (up to 5-6 hrs), regardless of when during this time the nuclei were inactivated by irradiation, the rate of respiration remains at its former level, characteristic of the first 6-7 hours of normal development. There is no increase in the rate of respiration after irradiation in the first period. This indicates that in this period the nucleus does not exert its control over the development of the respiratory apparatus, and the respiration of "anucleate" embryos (right until the time of death) remains the same as in the unfertilized eggs.

The controlling function of the nucleus begins only in the second period, at 5-6 hrs of development, and

continues until the 8-hr stage. The later the embryos were irradiated in this period, the further the respiratory apparatus continued to develop in the cells with inactivated nuclei. This means that the nucleus actively functions during this period and brings about an increase in respiration rate right up to the 12-hr stage (early gastrulation). If the eggs are irradiated at the beginning of the second period (5-hr stage), the respiratory apparatus remains at the same stage of development (5-6-hr stage) but irradiation at the end of the second period (8 hrs) permits the respiratory apparatus to continue its development up to the stage where the oxygen consumption would be equivalent to that at the 12-hr stage. In other words, during the three hours which constitute the second period, the functioning of the nucleus brings about a development of the respiratory apparatus in the cells for a period twice as long: from the 6-hr stage to the 12-hr stage. The ratio of the second rate to the first (3-hr to 6-hr) is 2 to 1 in the present case, and can be used as a measure of the degree of activity of the nucleus (see Fig. 7).

In the third period, as in the first, the degree of development of the respiratory apparatus does not depend on the time during the third period (from 8-12 hrs) at which the nuclei were inactivated by radiation. Whether the eggs were irradiated at the 8-hr stage or four hours later, the rate of respiration at the 12-hr stage was the same, characteristic for the 12-hr stage. It is clear that the nucleus did not exert its control in this period.

Finally, in the fourth period, as in the second, the dependence of further development on the time of irradiation during that period shows that the nucleus again exerts its control on the development of the respiratory apparatus.

Thus, each stage of development (but not the existence) of the respiratory apparatus is controlled by the activity of the nucleus, which is exerted either at a predetermined time or at almost the same moment. These relations to nuclear control are represented by narrow lines in Figure 6, which connect part of the rate-of-respiration curve with those stages (on the abscissa) in which the nuclei control the development of the respiratory apparatus in a given part of its development.

The results obtained show a periodicity in the functioning of the nuclei. This fact is not surprising at the present time, since a similar periodicity has already been established for nuclear control in relation to development and growth of the groundling embryo. Results are presented in Figure 7, for the sake of comparison, in which is also indicated the activity of the nucleus in relation to its control over growth and morphological features. The relation of the continuation of any period of development to the time when the nucleus, by its activity, brought about the completion of this period was used as a measure of its activity, as was noted above.

All three graphs (growth, morphological development and development of the respiratory apparatus) are in general very similar, though they differ somewhat in detail. It seems possible to conclude that in all three related processes, the nuclear control is exerted at approximately the same time. The dynamics of nuclear activity in relation to each of the three processes is, apparently, somewhat different. But it is still too early on the basis of these experiments to conclude that these

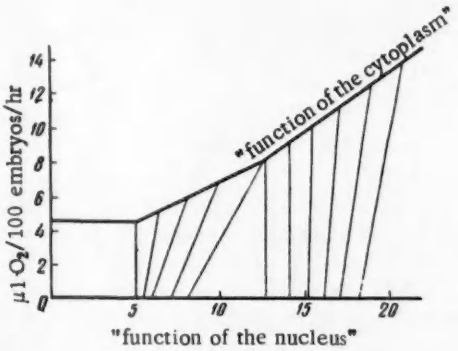

Fig. 6. The periods of activity of the nuclei, controlling development of the respiratory apparatus (abscissa), and the parts of the curve of respiration rate ("function of the cytoplasm") controlled by the nucleus.

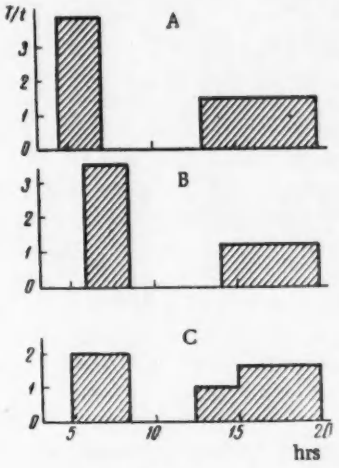

Fig. 7. Degree of nuclear activity controlling growth (A), morphological development (B), and development of the respiratory apparatus (C). Ordinate: The ratio of one hour of development (T = 1) to the time of nuclear activity (t) required to bring about a given stage of development (hours).

three functions of the nucleus are brought about independently.

The nature of nuclear control. The fact that cessation of increase in the rate of respiration always occurs significantly earlier than the cessation of development (Fig. 5), in some stages occuring almost immediately after the inactivation of the nucleus, makes it possible to state with sufficient certainty that the connection between nuclei and the development of the respiratory apparatus is closer than between nuclei and morphological features of development.

In the literature [19-21] it has been suggested that the nucleus might exert its control over respiration by virtue of the fact that the nucleus appears to be where respiratory coenzymes or hydrogen carriers, such as DPN and TPN, are synthesized. Can we suppose that in the present case we are dealing only with mechanism of a similar kind ? Probably not. On the other hand, the results that have been obtained indicate an increase in the number of mitochondria in the embryo [13, 22], along with an increase in the concentration of respiratory enzymes in the course of development [13, 23]. The control of rate of respiration through the level of DPN, if it generally functions in development, appears to be more rapid than some functional control, bringing about greater or lesser use of already existing physiological capacities. Perhaps the increase in the rate of respiration in some stages of development is brought about in exactly this way.

The nucleus controls the increase in the rate of respiration by means of the development of the whole respiratory apparatus of the cell, including the synthesis of enzymes, and formation of the structures of which it is composed. Thus, in this case as in all cases where analysis has been made of the mechanisms of control by which genotype is translated into the development of form and function, the role of the nucleus comes down to the synthesis of special respiratory enzyme proteins, mitochondrial structural proteins, and, perhaps, protein enzymes which bring about the formation of mitochondria, and the synthesis of their non-protein components. Some studies have indicated a direct participation of the cell nuclei in the formation of mitochondria [24-26].

It is not possible to judge on the basis of the results whether the synthesis of the proteins necessary for the formation of the respiratory apparatus occurs directly in the nucleus, or whether only the ribonucleic acid bases are synthesized in the nucleus, and then pass into the cytoplasm. However, it can be affirmed that, between the genetic elements of the nucleus that are destroyed by radiation and the process of increase in the rate of respiration during development, there exist a limited number of intermediary steps. That the number of steps is not large can be adduced from the fact that it is possible to stop the process almost instantly (by irradiating at 5-6 and 12-14 hrs of development). Besides this, the nucleus has the capacity to produce a "reserve", which at certain stages brings about an increase in the rate of respiration, after the nucleus has been inactivated, an extent equivalent to three hours of development (irradiation at 7-9 hrs).

A comparison of the results obtained by use of the method of radiation-inactivation of nuclei, for the investigation of morphological development and the development of the respiratory apparatus, makes it possible to think that in the present work a step has been made toward the understanding of the successive chain of processes in development. The further steps in this elucidation can be reached if even more basic features are chosen as criteria of the effects of radiation, for example: the formation of different parts of the respiratory system, the synthesis of different enzymes, and, perhaps, the synthesis of RNA.

SUMMARY

The rate of respiration in the embryos of the groundling remains constant up to the 5-7th hour of development (21.5°C), that is, to the middle blastula stage. The rate of respiration then suddenly increases, and over the course of embryonic development reaches 7-8 times its original level.

Ionizing radiation (40,000 r) does not decrease the rate of respiration, but quickly stops the increase in the rate, acting, therefore, not on the respiratory apparatus of the cell, but on the process of its development.

The unimpaired development of the respiratory apparatus in embryos with irradiated cytoplasm but intact nuclei (androgenetic haploids) and the cessation of development after irradiation of the nucleus show that suppression of the development of the respiratory apparatus by irradiation is brought about by the destruction of the nuclear apparatus which controls the increase in the rate of respiration during the course of development.

In irradiated embryos, the rate of respiration becomes constant within a short time, and remains at this level right up to the time when the embryo dies. A comparison of the stage of development to which the rate of respiration in irradiated embryos corresponds with the stage of development at the time of irradiation, shows that the cytoplasm possesses the capacity to increase the rate of respiration for some time without nuclear control.

An analysis of the dependence of the capacity of the respiratory apparatus to develop without nuclear control on the stage of development makes it possible to establish periods in which the nucleus exerts its control over the development of respiration. These periods closely parallel those of nuclear control over morphological development.

The chain of intermediate processes occurring between the nucleus and the development of the respiratory apparatus appears to be significantly shorter than that between the nucleus and the development of morphological features. The development of the respiratory

apparatus is considered as a model of cell differentiation in development. The possibilities of investigating the mechanisms of development by means of radiation inactivation of nuclei in this model are discussed.

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MUCOPOLYSACCHARIDES OF THE CHONDRAL AND BONE TISSUE IN THE PROCESS OF ONTOGENESIS AND REGENERATION

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Mucopolysaccharides, either free or bound to protein, are known to be a component of the basic structure of the various types of connective tissue, including the chondral and the osseous. Some authors attribute to polysaccharides a leading role in the structural organization of the basic structure; they are also considered to be essential components of the so-called "local factor" of calcification [1-4].

At present, we have basically at our disposal only indirect proof of the role of mucopolysaccharides in the life phenomena of chondral and bone tissue. It seems appropriate to conduct a study of the mucopolysaccharides of the bone tissue in the process of ontogenesis in order to achieve a deeper understanding of the biochemical processes occurring during the regeneration of bone tissue. Similar investigations were conducted by us earlier in a study of regeneration of muscular tissue and the formation of some types of connective tissue [5-8]. It is essential to investigate the mucopolysaccharide content not only in bone, but in cartilage as well, in view of the replacement of cartilage by bone in the process of development and regeneration.

The literature lists a number of papers devoted to biochemical studies of the mucopolysaccharide changes in cartilage with age, mainly the one of ribs [9-16]. It is established that the amount of polysaccharides in the cartilage decreases with age.

We know of only one preliminary report [17] dealing with the hexosamines of tubular bones in man; the author notes the gradual decrease in the amount of hexosamines during the entire lifespan of man. His investigations did not bring out any significant changes in the mucopolysaccharide content of human tubular bones in old age.

In the few existing investigations on this subject, the mucopolysaccharides were studied during the process of bone tissue regeneration mainly by means of histochemical and tagged inorganic sulfur methods. An intensive metachromasia was detected in the bone callus, indicating the presence of acid mucopolysaccharides [18]; it reached a maximum on the 7-10th day after fracture, and disappeared in the trabecules of the newly formed bone. A maximum inclusion of the tagged sulfate in the tissue of the callus was also observed on the 7-9th day [19-20]. A mucopolysaccharide was isolated from the tissues of the 5-7 day old callus, which resembled hyaluronic acid in its properties [21]. We do not know of any report on the quantitative content of mucopolysaccharides in bone callus.

METHODS

The study of the changes in the mucopolysaccharide content with age was conducted on human tubular bones and epiphyseal cartilage of the lower extremities (from 14 weeks of embryonic development to 83 years), as well as on tubular bones and epiphyseal cartilage of the legs and cranial bones (from 22 days of embryonic development to full growth age). In tubular bones the compact tissue from the diaphysial sections was the principal object of study, and in the epiphyseal cartilage, the sections which did not contain any visible mineral deposits. The cranial bones of rabbits were analyzed whole.

In rabbits, the amount of mucopolysaccharides in the bone callus was determined on the 7, 14, 21, and 55th day after the bones of one foreleg were fractured in a closed way under local anesthesia and sterile conditions, without subsequent application of a plaster of Paris cast. The amount of mucopolysaccharides was determined in whole callus tissues, as well as in bone fragments freed from callus tissues. The tibial bone of the symmetrical undamaged leg served as control. In view of the well-known morphological difference in the tissues of the bone callus, the average amount of mucopolysaccharides was calculated in every experiment as the mean of 6-8 parallel determinations. The material under investigation was carefully cleared from adjacent tissues, bone marrow and blood, was washed with cold water, comminuted, twice treated with acetone, and dried for an hour at 65-70°C, with subsequent storage at 0-2°C in a desiccator, over $CaCl_2$.

The amount of mucopolysaccharides was based on the quantity of hexosamines (the sum of glucosamines and galactosamines) in acid hydrolyzates of the tissues under investigation; the amount of hexosamines was determined by the method of Elson and Morgan [22] in Boas' modification [23], with some changes.

The aliquots of dried tissue (80 mg bone tissue, 25 mg cartilage) were decalcified for 18 hours in 1N HCl; the hydrolysis was performed with 2.5 ml of 4N HCl at

TABLE 1. Changes in the Hexosamine Content in the Bone and Cartilage Tissue of Man in Ontogenesis. (Hexosamine content in mg% of dry substance)

Age groups	Tubular bones			Epiphyseal cartilage		
	no. of cases	mean	fluctuation limits	no. of cases	mean	fluctuation limits
14-16th week of embryonic development	2	185	200—170	2	12 350	13450—11200
0-12 months	7	138	161—121	7	9 000	10400—7750
13 months - 5 years	5	125	133—116	5	7 450	8800—6650
6—10 years	1	105	—	1	5 600	—
11—20 "	3	96	99—88	3	5 300	7450—3050
21—40 "	2	98	103—94	1	5 900	—
41—60 "	4	98	108—79	2	3 950	4300—3600
61—83 years	8	94	104—88	2	4 400	5200—3300

100°C for a period of 8 hrs. After neutralization with 4N NaOH, the solutions were filtered and diluted with water to 10-25 ml, depending upon the hexosamine content. For the determination, 0.25 - 2 ml of the solution was taken, and the concentration of salts was brought to that of the standard solution by means of 4N NaCl. The standard solution contained 1 ml of glucosamine (0.2 mg of glucosamine), 1.5 ml 4N NaCl, and 0.5 ml water (total volume 3 ml). After adjusting the original volumes of the experimental solutions to 3 ml by means of water, there was added to each of the test tubes 1 ml of a 2% solution of acetylacetone (in 2N Na_2CO_3); the test tubes were heated for 45 minutes on a water bath at 89-92°C. After cooling, there were added 2.5 ml of 96% alcohol, 1 ml of Ehrlich's reagent (a 2.67% solution of n - dimethylaminobenzaldehyde, prepared in a mixture of equal parts of absolute alcohol and concentrated HCl) and the volume of the solution was made up to 10 ml by addition of alcohol. The developing pink color was determined after 2 hours in a photoelectric colorimeter with a green filter, with a blank containing all of the enumerated reagents, with the exception of glucosamine. The amount of glucosamines (the sum of glucosamines and galactosamines) was expressed in mg% of the dry weight of the tissue.

RESULTS

The amount of mucopolysaccharides in the tubular bones of man decreases with age. These changes are most pronounced during the embryonic period of development and during the first decade of life, at the end of which the hexosamine content reaches a magnitude which is characteristic for adults, and it remains at this level up to very old age. As can be seen from Table 1, the amount of mucopolysaccharides in the epiphyseal cartilage of man undergoes changes with age similar to the ones of the bone tissue, except that with advanced age the changes in the cartilage are somewhat more pronounced than in the bone.

It is of interest to note that in the epiphyseal lamella, e.g., in the zone of growth of the cartilage, there is observed at 12 years of age a higher hexosamine content (7450 mg%) as compared with the peripheral cartilage of the same epiphysis (4800 mg%), where the processes of growth are proceeding in a much less intensive way.

The decrease in the amount of mucopolysaccharides in ontogenesis has become still more evident from the study of tubular bones, and of the cranial bones of rabbits. To begin with, the last third of embryonic development (22 days), and up to the period of sexual maturity, the amount of hexosamines in the tubular bones of rabbits decreases approximately five times (see Table 2). The largest changes take place during the process of embryonic development and the first weeks of the postnatal period.

A similar dynamic in mucopolysaccharide content was observed by us also in the process of development of birds and guinea pigs. Thus, for instance, the hexosamine content of the bone tissue of a 14-day old chicken embryo amounts to 370 mg% of the dry tissue, of a three day old chick, 225 mg%, and of a sexually mature hen, 118 mg%.

In cranial bones of rabbits, which, unlike the tubular bones, develop directly from connective tissue, avoiding the cartilage stage, the hexosamine content is quite close to that of tubular bones (see Table 2) in absolute values, as well as in the character of changes with age. Only a slight lowering in hexosamine content (~20%) can be noticed in the epiphyseal cartilage of rabbits beginning with the 22nd day of embryonic development. It should be noted that during the indicated dates of embryonic development in rabbits the visible centers of ossification are still absent, and the cartilage fills out the whole epiphysis.

A histochemical study of the mucopolysaccharides in the process of development of bone tissue in man and some animals reflects the trend of changes revealed in the study of hexosamines. A substantial decrease of these components in the process of human development is evident (a pronounced metachromatic staining is changing to a slight one [8]).

In the process of bone tissue regeneration during the whole time interval (7-55 days after fracture) the

TABLE 2. Changes in the Hexosamine Content in the Bone and Cartilage Tissue of Rabbit in Ontogenesis. (Hexosamine content in mg% of dry weight)

Age, days	Tubular bones			Cranial bones			Epiphyseal cartilage		
	no. of animals	mean	fluctuation limits	no. of animals	mean	fluctuation limits	no. of animals	mean	fluctuation limits
Embryonic development									
22	12	520	550—490	12	415	460—370	14	8000	8000—8000
26	30	240	270—210	30	235	240—230	—	—	—
Postnatal development									
5	2	206	216—196	1	188	—	2	7100	7100—7100
14	2	149	159—138	2	159	150—141	2	6350	6300—6400
45—60	—	—	—	—	—	—	3	6500	7200—5200
Sexually mature	9	111	121—99	2	119	125—113	—	—	—

TABLE 3. Changes in the Hexosamine Content in the Process of Bone Tissue Regeneration in Rabbits. (Hexosamine content in mg% of dry weight)

Specimen	Days after fracture	No. of animals	Mean	Fluctuation limits
Bone callus	7	4	1760	1400—2000
Same	14	4	1050	580—1800
Same	21	4	730	420—1540
Same	55	2	145	134—165
Bone fragments	7—55	6	115	100—125
Symmerical bone	—	7	114	100—127
Normal bone	—	9	111	99—121

amount of hexosamines in well-cleaned bone fragments did not change, constituting, on the average, 115 mg% (Table 3).

At an early date, the hexosamine content of the bone callus differs sharply from that of the bone and its fragments, it being approximately 14 - 20 times higher than the bone hexosamine. Subsequently, a sharply pronounced decrease in the hexosamine content is noticed, with substantial fluctuations of the absolute content. Toward the 55th day, when the process of bone callus formation is essentially complete according to morphological and roentgenological data [24, 25], the hexosamine content at the place of injury is close to the values which are characteristic for mature bone tissue, amounting on the average to 145 mg%, with a small amplitude of fluctuation (134 - 166 mg%). Thus, during the course of regeneration from the 7th to the 55th day, after the fracture, the hexosamine content is reduced to one tenth to one fifteenth of the original value.

DISCUSSION

The decrease in the hexosamine content of bone tissue, which is observed during the process of ontogenetic development, and which reflects a change in the mucopolysaccharides, can be the result of various causes. The morphological rebuilding of the osseous tissue undoubtedly plays some role here, since it is known that the bone develops from cartilage tissue (tubular bones) or from connective tissue (the covering cranial bones) by way of gradual replacement of these. Since the mucopolysaccharide content in the bone tissue of adults is many times smaller than in cartilage and connective tissue, it is possible that the high content of these substances in bone at earlier stages of its formation is due to the presence of residual unabsorbed elements of chondral or connective tissue.

A lowering in hexosamine content may be also due to changes with age of the bone matrix itself.

Thus, it was shown in our laboratory by histochemical methods that the amount of mucopolysaccharides in the matrix of the bone decreases with growth. The bone tissue of human embryos is rich in acid mucopolysaccharides, the amount of which decreases sharply during the process of postembryonic development where the rough fibrous bone is being replaced by a laminated one, which is evidently linked with a decrease of amorphous

substance in the laminated bone Thus, the osseous tissue of a four month old fetus abounds in acid mucopolysaccharides, the amount of which decreases toward the age of two years, and continues to decrease in the subsequent period. Such changes are characteristic for the developing laminar structure [8]. It was simultaneously shown that in the cases where the amount of hexosamines in tubular bones exceeded 200 mg% they contained elements of cartilage tissue.

It has been established [9, 11, 12, 15, 16] that the decrease with age of the total amount of mucopolysaccharides in the rib cartilage is the result of two opposite processes, namely, a decrease in mucopolysaccharides containing uronic acids (probably chondroitinsulfate), and a rise in the amount of mucopolysaccharides lacking uronic acids (perhaps keratosulfates). This decrease in the amount of hexosamines in the cartilage is explained by the authors as being due either to a degeneration of chondrocytes [10] or to a lowering of the degree of polymerization of mucopolysaccharides [13], which facilitates their egress from the tissue. There are also reasons to presume that with age there also takes place a change in the ratio of various mucopolysaccharides which are a part of the ground substance of the cartilage [26].

In rabbits, as well as in man, the cartilage differs sharply from bones in the hexosamine content, the amount of which sharply decreases with age in both of these tissues. This applies also to the tubular and cranial bones of rabbits, in spite of the difference in their formation steps. There emerges also a difference in the formation of human and rabbit bone matrix of tubular bones along the embryogenetic stages. In man, the bone matrix is evidently formed mainly during the first half of intrauteral life, since toward this time the matrix differs only slightly in hexosamine content from the matrix of infants (Table 1). In rabbits, the process of cartilagenous matrix replacement takes place only during the second half of the gestation period, since towards the 22-26 days of embryonic development (the total duration of embryogenesis amounting to 30 days) the bone diaphysis contains a substantial amount of chondral tissue, and their hexosamine content exceeds several times the values which are characteristic for a fully formed osseous tissue.

Our results on the hexosamine content in human epiphyseal cartilage are in accord with some known data in the literature [12, 13].

The substantial variations in the mucopolysaccharide content of the osseous callus during the process of its regeneration, which was observed by us, is evidently due to the fact that various tissues, differing widely in their mucopolysaccharide content (nondifferentiated connective tissue, chondrous and osseous tissues) are participating in the formation of the callus. One should also take into consideration the fact that in various stages of regeneration the ratio of these tissues varies; where during the first stage of the regeneration process the callus consists mainly of soft tissues, there takes place during the subsequent building of the osseous callus the formation of bone tissue, with a corresponding decrease of mucopolysaccharides in the whole callus; their content in the still preserved sections of chondrous tissue is substantially higher as compared with the osseous elements. The large amplitude of fluctuations in the results obtained in parallel experiments can be attributed to the morphological heterogeneity of the various sections of the callus.

SUMMARY

It was revealed from the study of hexosamine content, which reflects the total amount of mucopolysaccharides in the tubular bones of man and rabbits, the cranial bones of rabbits, as well as the epiphyseal (joint) cartilage of man and rabbits, that the amount of hexosamines in cartilage surpasses by ten-fold their contents in bone tissue.

A notable decrease in hexosamines was observed during the process of ontogenetic development in man, and is especially pronounced at an age up to ten years; subsequently, up to very old age, the amount of these components either does not change (in bone tissue) or changes only in an insignificant way (in cartilage).

The bone matrix of human tubular bones takes shape, in respect to hexosamine content, during the first half of intrauteral life, and in rabbits, only toward the end of the embryonic period. The hexosamine content and the character of its changes in tubular bones and in the covering cranial bones of rabbits are quite close, in spite of the differences in the ways they are formed.

After fracture, the hexosamine content in the tissues of a bone callus reaches its maximal value in rabbits on the seventh day. During the process of regeneration, there is observed a decrease of hexosamines, and toward the 55th day, when the process of callus formation is basically completed (to judge from the morphological and roentgenological data), the hexosamine content approximates values which are characteristic for the osseous tissue of a sexually mature rabbit. These changes are evidently to a considerable extent linked to shifts in the structure of tissues composing the bone callus.

Of note is the similar direction in the quantitative changes in hexosamine in the process of ontogenetic development and in the regeneration of bone tissue as well. In both instances there is observed a parallelism between the changes in hexosamine content and the changes in mucopolysaccharides.

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NUCLEIC ACID CONTENT OF CELLULAR COMPONENTS OF THE MUCOSA OF THE SMALL INTESTINE AFTER γ-IRRADIATION

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At the present time it is established that ionizing radiation produces acute disturbances in DNA metabolism of radiation-sensitive tissues. Material with relation to RNA changes under the same conditions is more controversial. In the majority of works studying the content and rate of RNA replacement in irradiated animals, total RNA content without subdivision into fractions corresponding to its cellular distribution was discussed. In a number of publications, it was indicated that changes in RNA following irradiation of experimental animals were essentially the same as changes in DNA in sites such as the thyroid gland, spleen, bone marrow and mucosa of the small intestine [1-5]. Data concerning resistance to irradiation of RNA from tumor cells [6] and from embryonic tissues of the chicken [7] contradict the above data.

In view of the differences in qualitative composition [8] and metabolic activity of RNA from nuclei and cytoplasmic microstructures [9], the analysis of RNA changes produced by irradiation in the separate cellular microstructures is of interest; however, such data at the present time are limited.

In the work of Thompson and co-workers [10] on the nucleic acid content in the structural cellular components of rabbit bone marrow after local irradiation with γ-rays in the dose of 620 r, it was observed that within 48 hrs after radiation exposure, there was no change in DNA content calculated in terms of nuclear nitrogen; the amount of RNA was lowered only in the cytoplasm, primarily in the structureless hyaloplasm (64% of normal). Maxwell and Ashwell [11] have shown that seven days after total irradiation with x-rays in the dose of 640 r, there was a lowering of the nucleic acid content in all the cellular components of the mouse spleen. Other data discuss liver RNA metabolism, which in this organ presents a number of essential peculiarities.

Thus, the character of the reaction of RNA from cellular microstructures toward ionizing radiation, especially in its dynamics, is at present insufficiently clarified.

METHODS

The experiments were conducted on male rabbits weighing 2.5-3.5 kg, which were preliminarily starved for 18 hrs. The animals were investigated when normal and 2, 6 and 24 hours after irradiation with γ-rays from Co^{60} (1000-r doses). The dose strength was 490 r/min with the exception of two cases where the dose strength was 27 r/min. As has been established, the changes in dose strength within the limits of 13 to 630 r/min with all side radiation does not influence the effect of the harmful action under the condition of total irradiation. All of the experimental animals developed acute radiation sickness.

For the study of the quantitative distribution of RNA according to cellular microstructures, the mucosal tissue from the small intestine was utilized, since it is one of the radiation-sensitive tissues and at the same time it is morphologically more homogeneous than other tissues which are more susceptible to irradiation. This condition is of importance during differential centrifugation, for this process yields only neutralized data concerning components of heterogeneous cells of the studied tissue.

The mucosa of the small intestine was separated from the muscle layer, homogenized with ten volumes of 0.25 M solution of sucrose containing 0.0018 M $CaCl_2$ in a rapid blender rotating at 1000 rpm, and twice filtered through four and eight layers of gauze in order to remove tissue debris. The nuclei were separated from the cytoplasm by further centrifugation at 1200 rpm, and then they were washed with a sucrose solution with $CaCl_2$, and with a 0.1% solution of citric acid. Mitochondria and microsomes were precipitated from the supernatant fluid which remained after separation, as were nuclei (in centrifuges ASL-1 at 12,000 rpm and ASL-2 at 20,000 rpm respectively). All operations were conducted by the method of Hogeboom and Schneider as modified by Abelev [12]. At the end of the separation, the hyaloplasmic proteins (supernatant fluid after removal of mitochondria and microsomes) were precipitated with acid in a final

Nucleic Acid Content of Cellular Microstructures of the Mucosa of Rabbit Small Intestine (mg / g dry protein). Averages and Mean Errors of Four Experiments Involving Irradiation and Six Control Experiments.

Time after irradiation, hrs	Nuclei		Mitochondria	Microsomes	Hyaloplasm
	DNA	RNA	RNA	RNA	RNA
Normal	184,0±3,4	31,0±2,3	42,5±3,5	26,7±1,7	46,9±3,2
2 hrs after irradiation	146,1±7,8	31,5±0,6	38,1±3,0	28,4±1,3	48,2±2,8
6 hrs after irradiation	199,0±7,8	35,1±1,33	40,3±1,7	25,3±1,5	50,4±2,25
24 hrs after irradiation	133,0±6,9	37,0±1,46	44,0±1,4	23,5±1,6	43,0±2,9

Note. The change in DNA content 2 and 4 hours after irradiation was statistically significant ($p = 0.001$ and 0); that after 6 hrs was slightly significant ($p = 0.081$). Statistically significant changes in RNA content were absent (for nuclear RNA 24 hrs after irradiation, where the largest deviation from normal was observed, $p = 0.087$).

concentration of 2%. All of the isolated fractions, nuclei, mitochondria, microsomes and the hyaloplasmic proteins, were worked over successively with 2% perchloric acid, alcohol and an alcohol-ether mixture (1:1) in order to remove acid-soluble compounds and lipids. The protein-containing remnants were dried to constant weight in a vacuum desiccator at room temperature.

The quantitative determination of nucleic acids was conducted in batches of proteins after their extraction, with 10% perchloric acid at 70°, in accordance with the method described by Ceriotti [13]. DNA was determined by the colorimetric reaction with diphenylamine according to Dische [14]; RNA by the reaction with orcein according to Meibaum [15]. The optical density of the first reaction was measured with a photoelectric colorimeter, the second with an SF-4 spectrophotometer at 670 mμ.

Since the orcein reaction is not strictly specific for ribose, and produces color also with desoxyribose, all of the fractions were analyzed for their DNA content. For the determination of the true RNA content, the background formed by the observed quantities of DNA within the optical density of the orcein reaction was subtracted from the total optical density in the given reaction. In the nuclear fraction, for the purpose of control, separation of DNA and RNA according to the modified method of Schmidt and Tannehauser [16] was undertaken. In view of the fact that both methods gave similar results, subsequent determination of DNA and RNA was conducted without preliminary separation in the perchloric acid extract.

The results obtained in the present study are presented as average values from six experiments on normal rabbits and from four experiments on rabbits at different intervals after irradiation (in milligrams of nucleic acids per gram of dry protein mass in each fraction). Values for each experiment were obtained as an average of several parallel determinations.

RESULTS AND DISCUSSION

The results obtained are presented in the table. Attention is directed to the question of the equal distribution of RNA in microstructures of cells from small intestinal mucosa.

In analyzing the possibility of fraction contamination, it should be noted that under microscopic examination unbroken cells and mitochondria were not present in the nuclear fraction. The presence of some contamiation of the mitochondrial fraction by nuclear fragments was evidenced by the presence of DNA in this fraction. Smaller quantities of DNA were likewise observed in the microsome and hyaloplasm fractions, which could be explained by extraction of DNA from the nuclei and its absorption into the microsomes [17]. Isolation of nuclei and microstructures was conducted on one portion of the homogenate, which was prepared with 0.25 M sucrose and $CaCl_2$. At the same time it is known that $CaCl_2$ favors microsomal agglutination. For this reason, in the present experiments, the presence of part of the microsomes in the mitochondrial fraction was unavoidable. This was indicated by the large yield of the mitochondrial fraction and small yield of the microsomal fraction, as well as by the relatively high RNA content of the mitochondrial fraction.

Comparison of the quantitative content of nucleic acids in different microstructures of the small intestinal mucosa taken from normal and irradiated rabbits makes it clear that only the nuclear DNA was subject to change. Two hours after the action of γ-rays, the content decreased (from 184 mg/g dry protein mass to 146 mg) and it increased above normal (to 199 mg) after six hours. After 24 hours, a lowering of the DNA content (to 133 mg) was observed; this was more pronounced than that which occurred after two hours.

The RNA content in all four cellular fractions—nuclei, mitochondria, microsomes and hyaloplasm—was fairly

stable during all the intervals after irradiation and remained within the normal fluctuation limits. In twenty-four hours after irradiation, only the nuclear RNA content showed a tendency to be higher.

The changes in nuclear DNA content were of wave-like character. The intermittent increase in DNA content in the small intestinal mucosa of rabbits, six hours after irradiation, was evidently related to the disturbances in their nucleic metabolism. An analogous picture was brought out by Libinson [18] for bone marrow of rabbits after their irradiation with Co^{60} γ-rays in 1000-r doses: Four hours after irradiation, there was a decrease in DNA content; after eight hours there was an increase. Changes of the same nature were observed by Bishop and Davidson [19] for the DNA of the rabbit appendix.

The results demonstrating the constancy of the quantitative RNA distribution in different cellular microstructures from the small intestinal mucosa were somewhat unexpected. It has been established [4] that the incorporation of labelled C^{14}-glycine in purines of RNA from rabbit small intestinal mucosa was lowered to 70% five hours after irradiation with 800 r. The stability of the rabbit tissue RNA content toward ionizing irradiation is evidently peculiar to the tissue metabolism of rabbits. Thompson and co-workers [10] have explained the previous data concerning lowering of the RNA in bone marrow cells by changes in the cellular composition of the bone marrow and lowering in the number of immature forms (hyaloplasm of which is rich in RNA), and not by changes in the RNA content of the cells themselves.

The present data find support also in the results of Bishop and Davidson [19], who have established the absence of changes in phosphorus content of total RNA (or only small changes) from appendix and thyroid tissues of rabbits 1, 4, 8, and 24 hrs after irradiation with γ-rays in doses of 1000 r.

For this reason, the question concerning RNA changes under the influence of ionizing radiation cannot be answered in the same way for radiation-sensitive organs of different types of animals.

SUMMARY

Following the action of general irradiation with Co^{60} γ-rays in 1000-r doses, during the first days (2, 6, and 24 hours) after irradiation, changes of a cyclic nature were observed in the content of nuclear DNA of the mucosa of the rabbit small intestine.

Under the same conditions, the content of RNA in the nuclei, mitochondria, microsomes, and hyaloplasm of cells of the rabbit small intestine mucosa remained essentially unchanged.

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BIOCHEMICAL DIFFERENCES IN BOVINE SPERM AS A FUNCTION OF BREED AND SPERM QUALITY

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This study is concerned with the biochemical and physiological properties of sperm from bulls of different breeds. These properties are unquestionably of theoretical and practical interest, since they can serve as criteria of sperm quality, and especially of the viability of spermatozoids at 0° and -79°, temperature at which sperm to be used in artificial insemination is stored.

Thus far the subject of breed differences in sperm has received inadequate attention. Shergin [1, 2] has demonstrated that sperm from rams of different breeds differs in fructose content, in viability at 0°, and in fertilizing capacity. Flerchinger, Erb, et al. [3,4], in a study of the properties of Guernsey, Jersey, Holstein, and Hereford sperm, observed differences in sperm concentration, initial fructose level, lactic acid, and total and inorganic phosphorus. In a study of sperm of six breeds from Washington and Colorado, they found not only interbreed differences, but also differences between geographically separated individuals of a single breed. Evidently, interbreed differences are inherited, while differences of the second type are determined directly by the environment.

It is well known that the sperm of certain individuals cannot be stored at -79°; during freezing it succumbs to cold shock. The causes of this phenomenon have not as yet been determined.

De Groot believes [5] that loss of spermatozoid mobility during freezing can be attributed both to peculiarities of individual animals and to the quality of sperm of each ejaculate. Stamm [6] suggests that this is inherited. Several attempts have been made [7] to minimize cold shock by adjusting the cooling regime, and by changing the sperm diluent, but thus far with no success. Smirnov-Ugryumov [8] introduced phosphatide concentrates into the rations of animals whose sperm does not easily withstand freezing to -79° and obtained encouraging results.

Our investigations were designed to determine breed properties of sperm and to compare physiological and biochemical characteristics of sperm which can be successfully stored at -79° as against sperm which cannot be so treated.

METHODS

The studies were made at the Central Artificial Insemination Station in Moscow from May, 1958, through April, 1959. Sperm from 13 individuals of three breeds, Jersey (3), Kholmogor (4), and Black mottled (7), was studied. These are all milk breeds, although yield and butter fat content are different.

The animals were all maintained and fed in the same way. Food rations consisted of 6-10 kg of hay (winter) or 10-20 kg of grass (summer), and 3-5.5 kg of a mixture of grains (oats, barley, millet, wheat bran, sunflower oil cakes), fish oil, and sometimes eggs.

The chief material used for the study was ejaculate which had been concentrated two-fold. The following physiological and biochemical determinations were made: activity of spermatozoids under a microscope, according to the ten-point system; speramatozoid resistance to 1% NaCl; percent of living spermatozoids, as measured by staining in 5% aqueous eosin; sperm concentration, as measured in a Goryaev chamber; dehydrogenase activity, as measured by decoloration of methylene blue; pH; initial fructose level, according to Rowe-Bishop [3, 4]; fructolysis index (quantity of fructose decomposed in 15 minutes by 1 ml of sperm at 37°, expressed on the basis of 10^9 spermatozoids); P_{total}, according to Fiske-SubbaRow [10]; P_{lipid} and N_{total}, according to the Kjeldahl method [10]; calcium, as determined by trilonometric method; absolute viability according to [9]. Resistance of spermatozoids to cold shock was determined according to their movement after a 15-minute exposure of 0.3 ml of undiluted sperm in a test tube to a 0° temperature. Determination of movement was also made after a 24-hour exposure of diluted sperm to a -79° temperature (dry ice). All material was subjected to variational analysis.

RESULTS

Data on the biochemical and physiological characteristics of bovine sperm are presented in Table 1.

Jersey sperm differs markedly from sperm of the other two breeds in the overwhelming majority of

characteristics. As a rule, its characteristics indicated a greater biological stability as compared with Kholmogor and Black mottled sperm. The volume of the first and second ejaculates was smaller, and the dehydrogenase activity was higher. As is well known, in spermatozoids respiration is a most important vital process, furnishing 9/10 of the total energy requirement. Ability to reduce methylene blue has been proposed by Shergin [1] as a measure of sperm quality in bulls and rams. It has been found that the more vigorous the oxidation – reduction processes, the higher the sperm quality. This characteristic is therefore very important in an evaluation of sperm quality.

Resistance of Jersey sperm to 1% NaCl was higher than for the other two breeds. The sperm was more concentrated and more acid, which is due to the fact that during ejaculation there is less dilution by alkaline secretions of the accessory glands and that the accumulation of lactic acid during fructolysis in the thick sperm is more rapid. In this case the spermatozoids rapidly develop a condition of acid anabiosis, and as a result the period during which they can be used in artificial insemination is extended. The fructolysis index was lower in Jersey sperm, and N_{total}, P_{total}, and P_{lipid} were considerably higher.

The important role of phosphorus in formation of high-energy bonds and the exclusive dependence of biochemical and physiological processes in the spermatozoids on phosphate metabolism should be emphasized in this connection. The high P_{lipid} content indicates an abundance of acetal phosphatides, which favors a long period of viability at low temperatures. The average absolute viability for Jersey sperm was 98.6 and for Kholmogor and Black mottled sperm 94.2 and 74.8, respectively. Jersey sperm proved to be more resistant to cold shock. Sperm activity (by relative grade) after 24 hours storage at -79° was 0.3 for Jersey sperm, 0.25 for Kholmogor sperm, and 0.18 for Black mottled sperm.

Jersey sperm did not differ greatly from Kholmogor sperm with respect to number of living spermatozoids, initial fructose level, amount of calcium, or activity after equilibration in glycerin prior to freezing, but it did differ markedly from Black mottled sperm. The number of living spermatozoids was higher, the calcium and fructose levels lower, and the activity after equilibration higher. The lower initial fructose level in Jersey sperm should not be considered disadvantageous; on the contrary, it prevents the accumulation of lactic acid, which is injurious to spermatozoids.

Kholmogor sperm differed from Black mottled sperm in that the volume of both the first and second ejaculates was lower, and spermatozoid concentration was also lower. However, the percentage of spermatozoids which did not stain in eosin (living) was higher as was the resistance to 1% NaCl. P_{total} levels were higher, absolute viability was greater, initial fructose and calcium levels were lower, and activity both after equilibration and after freezing (-79°) was greater in Kholmogor sperm. There were no differences between the two breeds with respect to dehydrogenase activity, cold shock, N_{total} and P_{lipid} content, and fructolysis index.

These two breeds differ from each other to a smaller extent, therefore, than from Jersey.

Our results have shown that Jersey sperm is well able to withstand freezing to -79°; Kholmogor sperm is somewhat less resistant to storage at -79°, while Black mottled sperm is least resistant to such treatment. As indicated by the foregoing, several of the biochemical characteristics of Jersey sperm are in theory more favorable to viability, and indeed Jersey sperm shows great biological stability under unfavorable conditions.

Thus, the most important criterion of biological stability (ability to undergo storage at -79°), which is of enormous practical significance, is related to a whole series of biological and biochemical characteristics (initial fructose level, calcium, P_{total}, and P_{lipid} levels, fructolysis, viability at 0°). It seems likely that this is not a fortuitous circumstance. In order to determine whether there is any relationship between viability at -79° and the characteristics indicated, we divided the sperm into two classes: that which undergoes freezing without injury, and that which cannot easily be stored at low temperature.

These two types of sperm are compared in Table 2. Sperm which iswell able to withstand a -79° temperature is characterized by lower ejaculate volumes, lower initial fructose, and calcium levels, higher P_{total} and P_{lipid} contents, greater viability at 0°, and greater activity after equilibration in glycerin. It is also better able to withstand cold shock, has a higher percentage of individuals which do not stain in eosin, a higher concentration, greater resistance to 1% NaCl, and higher dehydrogenase activity.

The fructolysis index of such sperm is lower than that of sperm which is not easily stored at low temperatures. It is apparent that the more slowly fructose is degraded,the more easily the sperm is stored, since under these conditions the surrounding environment is less acid. There were no significant differences between the two groups with respect to N_{total} content.

From the foregoing it can be concluded that capacity for storage at low temperature is related primarily to such things as initial fructose content, fructolysis index, content of P_{total}, P_{lipid}, and calcium, and absolute viability. (Sperm is more resistant to cold shock the lower the calcium level, the initial fructose level, and the fructolysis index, the higher the P_{total} and P_{lipid} levels, and the greater the absolute viability.) It should be pointed out that Jersey sperm possesses in greater degree those characteristics associated with the ability

TABLE 1. Biochemical and Physiological Differences in Bovine Sperm as a Function of Breed

Breed	Sperm volume, ml		Time for decoloration of methylene blue, min.	Living sperm which do not stain with eosin, %	Concentration, billions	Resistance, thousands	pH	Initial fructose level, mg %	Fructolysis index, mg	N_{total}, mg %	P_{total}, mg %	P_{lipid}, mg %	Calcium, mg %	Response to cold shock by rel. grade*	Absolute viability	Activity after equilibration, by rel. grade	Activity after 24 hours' storage at -79°, by rel. grade
	1st ejaculate	2nd ejaculate															
Jersey	3.3 ±0.2	3.4 ±0.1	10 ± 0.9	76 ±1	1.1 ±0.04	32 ± 2	6.5 ±0.03	416 ±52	1.4 ±0.2	1112 ± 35	129 ± 6	12.5 ±0.7	25.6 ± 2.4	0.15	98.6 ±6	0.5 ±0.02	0.3 ±0.02
Kholmogor	3.7 ±0.2	4.1 ±0.2	13 ± 1	74 ±1	0.9 ±0.04	28 ± 1	6.6 ±0.02	390 ±41	1.6 ±0.3	1047 ± 50	97 ± 5	9.7 ±0.6	23.2 ± 2.7	0.07	94.2 ±5	0.5 ±0.02	0.25 ±0.02
Black mottled	4.3 ±0.2	4.2 ±0.3	12 ± 0.8	72 ±1	1.0 ±0.04	21 ± 1	6.6 ±0.02	556 ±55	1.6 ±0.2	1069 ± 36	95 ± 4	9.2 ±0.5	29.1 ± 2.1	0.07	74.8 ±4.9	0.4 ±0.02	0.18 ±0.01

* These data were not statistically treated.

TABLE 2. Biochemical and Physiological Differences in Sperm as a Function of Quality

Group	Amount of Sample	Sperm volume, ml		Time for decoloration of methylene, blue min	Living sperm which do not stain with eosin, %	Concentration, billions	Resistance, thousands	pH	Initial fructose level, mg %	Fructolysis index, mg	N_{total}, mg %	P_{total}, mg %	P_{lipid}, mg %	Calcium, mg %	Response to cold shock by rel. grade*	Absolute viability,	Activity after equilibration, by rel. grade	Activity after 24 hours' storage at -79° by rel. grade
		1st ejaculate	2nd ejaculate															
Sperm highly resistant to storage at -79° for 24 hours	37	3.6 ±0.1	3.8 ±0.1	11 ± 0.5	72 ±1	1.1 ±0.02	30 ±1.7	6.5 ± 0.02	420 ± 32	1.3 ±0.2	1025 ± 36	117 ± 5	11.9 ± 0.6	23.6 ±2.5	0.09	104.6 ± 3.5	0.5 ±0.01	0.32 ±0.01
Sperm poorly resistant to storage at -79° for 24 hours	39	4.2 ±0.2	4.0 ±0.2	12 ± 0.9	71 ±1	1.06 ±0.02	25 ±2.1	6.5 ± 0.02	470 ± 44	1.80 ±0.2	1017 ± 39	95 ± 3	9.2 ± 0.4	27.0 ± 1.7	0.08	70.1 ±4.5	0.4 ±0.02	0.15 ±0.009

* These data were not statistically treated.

to withstand freezing than does sperm of the other two breeds. Such characteristics are evidently indications of great biological vigor in their possessors.

SUMMARY

It was shown that there are biochemical, physiological, and biological differences among sperm from bulls of different breeds.

Jersey sperm differs from Kholmogor and Black mottled sperm in the following ways: smaller volume of first and second ejaculates, higher dehydrogenase activity, higher percentage of living spermatozoids, higher sperm concentrations, higher resistance to 1% NaCl, lower pH, lower fructolysis index, higher levels of total and lipid phosphorus, greater viability at 0°. With respect to initial fructose content, calcium content, and activity after equilibration, it does not differ substantially from Kholmogor sperm, but it does differ markedly from Black mottled sperm. Kholmogor sperm differs from Black mottled sperm in the following ways: smaller volume of first and second ejaculates, lower sperm concentration, lower fructose content, lower calcium content, higher percentage of living spermatozoids, greater amounts of total and lipid phosphorus, and greater absolute viability.

Jersey sperm is more resistant to cold shock and is well able to withstand freezing to -79°, its activity after thawing being 0.3. Kholmogor and Black mottled sperm is less resistant to cold shock and is less able to withstand freezing, the activities after thawing being 0.25 and 0.18 respectively.

Apart from breed differences, sperm which withstands freezing differs from that which is not easily stored at low temperature in that it has a lower initial fructose level, a lower calcium content, a lower fructolysis index, and a higher total and lipid phosphorus content. These characteristics should be regarded as related in some way to the greater ease of storage at low temperature.

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A SIMPLE METHOD OF SCREENING FOR ANTIMETABOLITES

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The term antimetabolite refers to structural analogues of metabolites which interfere with the functioning of said metabolites in biochemical reactions. It is characteristic of inhibition by antimetabolites that it is relieved by corresponding amounts of metabolites. It is well known that many antimetabolites have a chemotherapeutic value, and therefore it is very important to search out new ones.

The methods employed in the study of antimetabolites are extremely varied; microbiological methods are often used for this purpose.

Hitchings et al. [1, 2] performed a number of studies on antagonists of purine and pyrimidine bases of nucleic acids using Lactobacillus casei grown on a complex synthetic medium. There are several other studies devoted to this subject.

In previous investigations we used a culture of Escherichia coli (strain 355) growing on a simple glucose saline medium to study antagonism phenomena [3-6]. In this study the same strain is used to screen purine derivatives for antimetabolite activity. To check whether this is a suitable test organism, the effect of certain well-known antimetabolites of the purine series which have found use in the treatment of malignant tumors (6-mercaptopurine, 2-amino-6-mercaptopurine, 8-azaguanine) was studied. As controls, antitumor agents which are not purine antagonists (thiophosphamide, colchamine, mileran, embichin, aminopterin, 4-dimethylamino pteroyl glutamic acid) were used.

METHODS

The test organism was strain 355 of E. coli grown on ordinary agar which was sown in glucose – saline medium prior to the experiment.

Composition of the glucose – saline medium was as follows: Na_2SO_4 – 11.35 g, NH_4Cl – 5 g, K_2HPO_4 – 4 g, $MgSO_4$ – 0.4 g, More's salt – 0.015 g, glucose – 10 g, glass-distilled water – 1 liter, adjusted with 4% NaOH to pH 7.6. Five-ml portions of medium were poured into test tubes and then sterilized with live steam.

In order to prevent the introduction of significant amounts of metabolites with the medium and with dead bacteria, a minimum amount of inoculum was used. Experiments showed that an inoculum of 4000 bacteria per 5 ml of medium consistently produced growth.

The bacterial culture in glucose – saline medium was first washed by centrifugation to remove metabolites and was then suspended in an amount of medium to give a concentration of 4000 bacteria per 0.1 ml. To each test tube was added 0.1 ml of suspension.

Growth was determined after 1, 2, 3, 4, and 5 days according to suspension tubidity. Threshold concentrations of materials tested at which growth was absent were recorded. In the description of the results, the concentrations designated are those which caused growth inhibition within 20-24 hours after inoculation. (Poorly soluble materials were used as suspension.)

Experiments showed that mileran (myelosan) gave

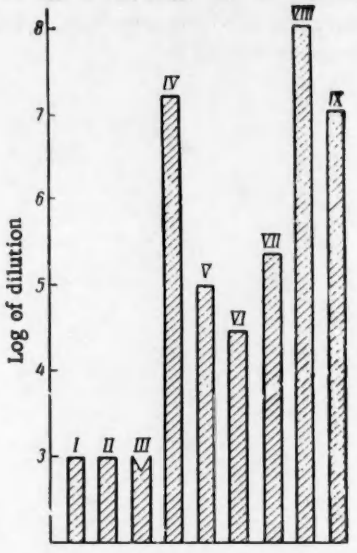

Fig. 1. Minimal concentrations of anti-tumor agents which produced growth suppression in E. coli. I) Thiophosphamide; II) colchamine; III) myelosan; IV) embichin; V) aminopterin; VI) 4-dimethylamino pteroyl glutamic acid; VII) 8-azaguanine; VIII) 6-mercaptopurine, IX)2-amino-6-mercaptopurine.

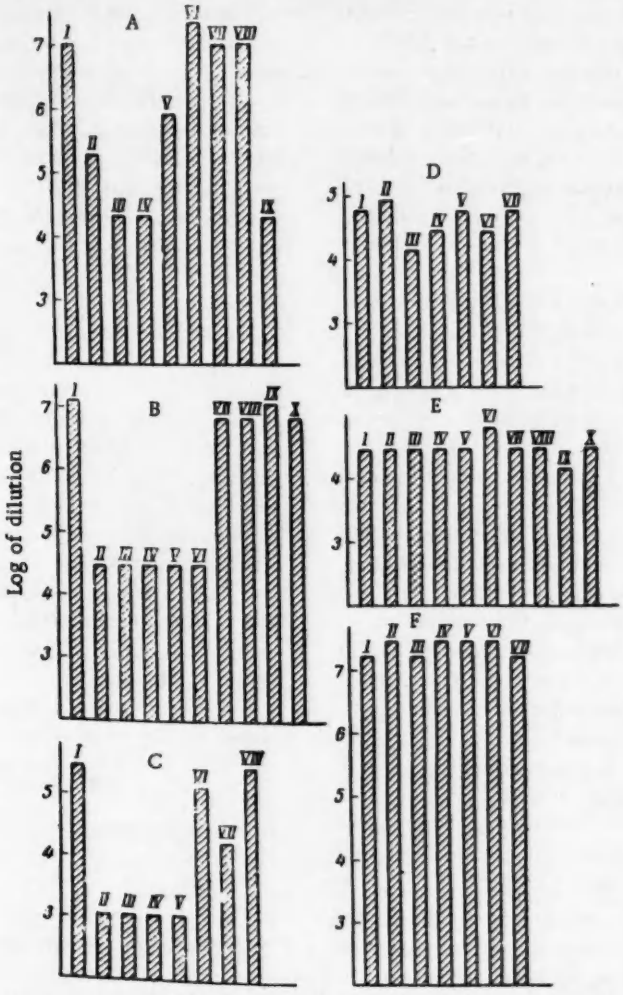

Fig. 2. The effect of purine and pyrimidine bases on the antibacterial activity of antimetabolites. A) 6-Mercaptopurine; I) 6-Mercaptopurine; II-IX) the same with the addition of: II) Guanine;III) adenine; IV) hypoxanthine; V) xanthine; VI) thymine; VII) uracil; VIII) cytidine; IX) guanosine. B) 2-Amino-6-mercaptopurine; I) 2-Amino-6-mercaptopurine; II-X) the same with the addition of: II) guanine; III) adenine; IV) xanthine; V) hypoxanthine; VI) guanosine; VII) uracil; VIII) thymine; IX) cytidine; X) orotic acid. C) 8-Azaguanine; I) 8-Azaguanine; II-VIII) the same with addition of: II) adenine; III) guanine; IV) hypoxanthine; V) guanosine; VI) uracil; VII) xanthine; VIII) thymine. D) Aminopterin; I) Aminopterin; II-VII) the same with the addition of: II) adenine; III) guanine; IV) uracil; V) thymine; VI) hypoxanthine; VII) xanthine. E) 4-Dimethylamino pteroyl glutamic acid. I) 4-Dimethylamino pteroyl glutamic acid; II-X) the same with the addition of: II) guanine; III) adenine; IV) hypoxanthine; V) xanthine; VI) uracil; VII) thymine; VIII) orotic acid; IX) guanosine; X) cytidine. F) Embichin; I) Embichin; II-VII) the same with the addition of: II) guanine; III) adenine; IV) hypoxanthine; V) xanthine; VI) uracil; VII) thymine.

practically no growth inhibition, colchamine and thiophosphamide gave inhibition only at very high concentrations, and the remaining materials tested gave inhibition at fairly high dilutions. A day after inoculation, when the control showed good growth, aminopterin and 4-dimethylamino pteroyl glutamic acid produced inhibition at a concentration of 1 : 64,000. The corresponding concentrations for 8-azaguanine, 2-amino-6-mercaptopurine, and 6-mercaptopurine were 1 : 256,000, 1 : 12,800,000, and 1 : 131,072,000 and for embichin, 1 : 16,384,000 (Fig. 1.).

Those materials which did not inhibit growth or which inhibited it only at very high concentrations (thiophosphamide, colchamine, myelosan) are evidently not antimetabolites or at least they do not manifest their antimetabolite property on bacterial cultures. The other materials may or may not be antimetabolites. To determined whether a given material is an antimetabolite, experiments on antagonism were set up. In these experiments the bacteriostatic activity of a presumptive antimetabolite was determined on basic medium and on a medium with a particular metabolite added.

Results are presented in Fig. 2, in which growth suppression produced by antitumor agents alone is compared with that where an antagonist is present. Growth suppression is expressed as the negative logarithm of the dilution of the antitumor agent.

Experiments with 6-mercaptopurine showed that its effect is sharply reduced in the presence of guanine, adenine, and especially hypoxanthine, and is almost unchanged in the presence of xanthine, thymine, and uracil (Fig. 2, A).

The action of 2-amino-6-mercaptopurine is antagonized by hypoxanthine, adenine, guanine, guanosine, and xanthine, while thymine, cytidine, and orotic acid have no antagonistic effect (Fig. 2, B).

Hypoxanthine, guanine, and adenine are highly effective antagonists of 8-azaguanine, while xanthine is considerably less effective. Uracil and thymine are inactive in this respect (Fig. 2, C).

The activities of aminopterin, 4-dimethylamino-pteroyl glutamic acid, and embichin are unchanged in the presence of purine and pyrimidine bases (Fig. 2, D, F, F). As is well known, 4-dimethylamino pteroyl glutamic acid and aminopterin are antagonists of folic acid [7].

Thus, 6-mercaptopurine, 2-amino-6-mercaptopurine, and 8-azaguanine are antagonized by purine bases, the most effective being hypoxanthine, adenine, and guanine.

It may therefore be concluded that the former are true antimetabolites. This conclusion is in basic agreement with published information, and the method of screening for antimetabolite activity which has been described may therefore be regarded as an appropriate one.

SUMMARY

Antimetabolites interfering with purine metabolism such as 6-mercapto purine, 2-amino-6-mercaptopurine, and 8-azaguanine inhibit growth of Escherichia coli on a glucose-saline medium. This growth inhibition is relieved by several purine bases (hypoxanthine, adenine, guanine). Considerable growth inhibition is caused by aminopterin, 4-dimethylamino pteroyl glutamic acid and embichin on a glucose-saline medium, but this is not affected by purine bases.

Colchamine and thiophosphamide inhibit growth of E. coli on a glucose-saline medium only in very high concentrations, whereas myelosan does not cause any inhibition of growth.

It is concluded that cultivation of E. coli on a glucose-saline medium may be used to screen for antimetabolites.

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THE POLAROGRAPHIC DETERMINATION OF DINITROPHENYL AMINO ACIDS

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In recent years the use of the method of forming dinitrophenyl derivatives of amino acids (DNP-amino acids) has been widely used for determining the N-terminal amino acids of proteins and peptides [1, 2]. It has also been used for amino acid analysis of protein hydrolyzates [3-6], and in the determination of C-terminal amino acids of hydrazinolysis [7, 8].

In all the uses of this method the need arises for the quantitative determination of the DNP-amino acids after their chromatographic separation. A number of methods have been suggested for the determination of DNP-amino acids. Of these, colorimetric analysis [1], determination of nitro groups by titration with $TiCl_3$ and [3], determination of the amount of hydrogen used in their hydrogenation [9] are suitable for determination of relatively large amounts of DNP-amino acids and can be used only after their separation by column chromatography. However, at the present time paper chromatography is chiefly used for the separation of DNP-amino acids since it assures great ease and reliability in their identification and permits work with the least amount of starting material. In this case, the only sufficiently sensitive method for the quantitative determination of DNP-amino acids is the spectrophotometric determination by the absorption at 360 mμ [2].

As is known, nitro compounds are reduced well on a dropping mercury electrode. Considering the high sensitivity of the polarographic method, we can assume that polarographic determination of the DNP-amino acids is also a sufficiently suitable method for their determination. The only DNP-amino acid already studied polarographically is di-DNP-histidine. Wenger and co-workers [10] worked out a method for the polarographic determination of histidine in the form of di-DNP-histidine in the absence of other amino acids. However, as will be shown below, the other DNP-amino acids differ sharply in their polarographic behavior from di-DNP-histidine.

The problem of the present work was to develop a method for polarographic determination of DNP-amino acids after their separation by paper chromatography.

METHODS

DNP-amino acids were synthesized by the method of Abderhalden and Blumberg [11] and of Sanger [1]. The resulting products were twice recrystallized. The melting points of all the DNP-amino acids, except for DNP-glutamic acid which we could not obtain in the crystalline state, corresponded to the literature data [1]. The purity of the DNP-amino acids was also tested chromatographically. The use of our method for chromatographic separation of DNP-amino acids was described in detail earlier [12].

The work was carried out at first in an SGM-8 polarograph of the "Geologorazvedka" factory, and then (in most

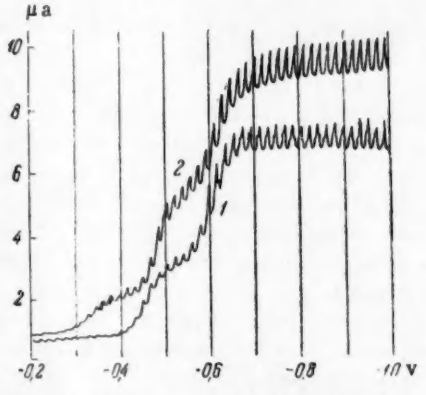

Fig. 1. Polarograms of DNP-amino acids. 1) DNP-alanine; 2) di-DNP-histidine, pH 8.2; concentration $1.7 \cdot 10^{-4}$ M.

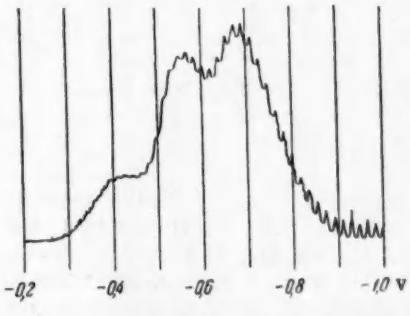

Fig. 2. Complete differential polarogram of di-DNP-histidine pH 8.2; concentration $1.7 \cdot 10^{-4}$ M; scale of differentiation - 1 .

TABLE 1. Dependence of Diffusion Current on Concentration for DNP-glycine

Concentration, mmole/liter	i_d, μa	i_d/c	Concentration, mmole/liter	i_d, μa	i_d/c
1,005	22,7	22,6	$2{,}0\cdot10^{-2}$	0,455	22,8
$8{,}39\cdot10^{-1}$	17,9	21,4	$1{,}67\cdot10^{-2}$	0,368	22,0
$6{,}70\cdot10^{-1}$	14,9	22,3	$1{,}33\cdot10^{-2}$	0,29	21,8
$5{,}02\cdot10^{-1}$	11,2	22,3	$1{,}0\cdot10^{-2}$	0,222	22,2
$3{,}35\cdot10^{-1}$	7,35	22,0	$6{,}7\cdot10^{-3}$	0,152	22,7
$2{,}51\cdot10^{-1}$	5,72	22,8	$5{,}0\cdot10^{-3}$	0,115	23,0
$1{,}68\cdot10^{-1}$	3,80	22,6	$3{,}33\cdot10^{-3}$	0,083	24,9
$1{,}26\cdot10^{-1}$	2,80	22,2	$2{,}50\cdot10^{-3}$	0,050	20,0
$8{,}4\cdot10^{-2}$	1,90	22,6	$1{,}67\cdot10^{-3}$	0,039	23,4
$6{,}3\cdot10^{-2}$	1,40	22,2	$1{,}59\cdot10^{-3}$	0,038	23,9
$4{,}0\cdot10^{-2}$	0,93	23,2	$1{,}19\cdot10^{-3}$	0,026	21,8
$3{,}0\cdot10^{-2}$	0,69	23,0			
$2{,}67\cdot10^{-2}$	0,64	24,0			

Average: $22{,}5\pm0{,}56$*

*Calculated for concentrations exceeding $5\cdot10^{-6}$ M.

experiments) on an electron integro-differentiating PE-312 polarograph. The polarographic cell with an external anode (saturated calomel half cell) was described by Sochevanov [13] and permitted carrying out determination in 0.5-1 ml of solution. The cell was kept thermostatically at 25° ± 0.1°. The dropping period of the capillary was determined at a potential of 0.8 v at which a plateau of the diffusion current was reached. Most of the work was carried out with a capillary for which m = 0.87 mg/sec, t = 3.8 sec, $m^{2/3} \cdot t^{1/6} = 1.138$. As a base we used a Britton-Robinson buffer solution or a borate buffer with pH 8.2. Oxygen was removed from the solution by adding Na_2SO_3.

Maxima on the polarogram were suppressed by those surface-active substances which were washed from the agar-agar bridge connecting the outer anode with the cell. In using an inner anode it was necessary to add a solution of gelatin.

RESULTS

All of the studied mono-DNP-amino acids, and also a di-DNP-lysine, give two waves in polarography. A typical polarogram is shown in Fig. 1 (polarogram 1). The limiting current is proportional to the square root of the height of the mercury column, which indicates its diffusion character. For all the DNP-amino acids the value of $E_{\frac{1}{2}}$ is practically the same and shifts toward the negative side with rising pH (at pH 8.2 $E'_{\frac{1}{2}} = -0.47$ v and $E''_{\frac{1}{2}} = -0.62$ v). The height of the wave does not depend on pH. An exception is DNP-aspartic acid, in which the height of the second wave decreases at pH > 9.

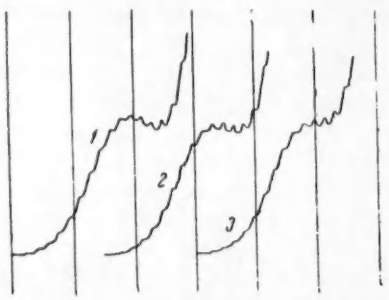

Fig. 3. Initial part of the differential polarogram of di-DNP-histidine in the presence of DNP-leucine. pH 8.2; polarogram begins from -0.2 v; abscissa through 0.1 v; scale of differentiation -1; 1) di-DNP-histidine, concentration $2.34\cdot10^{-4}$ M; 2) and 3) the same + DNP-leucine in concentrations of $1.15\cdot10^{-2}$ and $1.84\cdot10^{-2}$ M respectively.

Since the two waves are not sufficiently clearly separated from each other, it is more suitable for analytical purposes to measure the total diffusion current. The determination of the DNP-amino acids is best carried out at pH 8.0-8.5, since they are sufficiently soluble at this pH value and are easily washed from the paper. The use of a solution with a higher pH value is not desirable, since it might lead to partial decomposition of the DNP-amino acids, or a fall in the diffusion current in the case of DNP-aspartic acid.

Determination of the dependence of diffusion current on concentration was carried out in most cases in concentrations from $5\cdot10^{-6}$ M to $2\cdot10^{-4}$ M, and for DNP-glycine and DNP-serine, from 10^{-6} M to 10^{-3} M. For all the DNP-amino acids studied, the value of the diffusion current was directly proportional to the concentration. Examples are given in Table 1.

From the data for all the DNP-amino acids studied we calculated the diffusion current constant $K = i_d/cm^{2/3} \cdot t^{1/6}$ (Table 2). These values, as is known, do not depend on the capillary used and are characteristic for each substance studied polarographically [14]. For some DNP-amino acids the determination of the dependence of the diffusion current on concentration was repeated with the use of different capillaries ($m^{2/3}t^{1/6}$ from 1.14 to 2.09). On this, the value of K remained constant within the limits of error of the experiment. DNP-proline, DNP-aspartic acid, DNP-glutamic acid, DNP-valine, and DNP-leucine give almost the same values for K, which permits us to construct a general calibration curve for them.

TABLE 2. Diffusion Current Constants of Some DNP-Amino Acids

DNP-amino acid	K	Mean square error,%	DNP-amino acid	K	Mean square error,%
DNP-glycine	19,8	2,5	DNP-aspartic acid	17,1	3,6
DNP-alanine	18,9	2,5	DNP-glutamic acid	16,8	2,8
DNP-serine	18,9	2,7	DNP-valine	16,9	2,9
DNP-threonine	18,4	3,1	DNP-leucine	16,8	3,4
DNP-phenylalanine	17,9	3,3	di-DNP-lysine	33,0	3,3
DNP-proline	17,1	2,6	di-DNP-histidine	24,1	3,3

TABLE 3. Results of Polarographic Determination of DNP-Amino Acids

DNP-amino acid	Taken μg	Found μg	Error, %	DNP-amino acid	Taken μg	Found μg	Error, %
DNP-glycine	40,5	40,4	—0,2	DNP-aspartic acid	15,2	15,3	+0,7
"	10,1	10,38	+1,7	"	10,3	10,4	+1,0
"	2,40	2,32	—3,3	"	10,1	9,8	—3,0
DNP-serine	17,6	17,7	+0,6	"	5,15	4,95	—2,0
"	4,10	4,01	—2,2	DNP-valine	5,76	5,62	—2,4
DNP-alanine	8,52	8,80	+3,2	"	4,72	4,80	+1,7
DNP-leucine	7,28	7,41	+1,8	"	1,94	2,01	+3,6

The accuracy of the analysis is characterized by the value for the mean square error in determination of K (Table 2), and also by the results of determination of known amounts of DNP-amino acids (some of these are given in Table 3).

As the data show, the relative error in determination of the DNP-amino acids at concentrations of from $5 \cdot 10^{-6}$ M to $2 \cdot 10^{-4}$ M does not exceed 4%. In a volume of solution equal to 1 ml, these concentrations correspond to about 1.5 to 60 μg of DNP-amino acid (the molecular weight of most amino acids is close to 300). The amount of DNP-amino acid which can be determined after paper chromatographic separation is usually within these limits. However, it is possible to determine the DNP-amino acids polarographically at concentrations to 10^{-6} M, that is, to 0.3 μg/ml. The relative error then increases and reaches 10% (Table 1).

On the basis of the results which we obtained, we have worked out the following method for the polarographic determination of the DNP-amino acids separated on a paper chromatogram. The spot of DNP-amino acid is cut from the chromatogram and cut up into fine pieces. These pieces are placed in a small test tube and exactly 1 ml of buffer solution with pH 8.2-8.3 is added; the tube is allowed to stand for 20 min, shaking once or twice. (It was shown by experiment that when the DNP-amino acids were washed out by such a solution at room temperature, complete washing out was obtained after 15-20 min.) After 20 min the solution was poured into a polarographic cell (the fibers and pieces of paper do not interfere) and a pinch (10-20 mg) of Na_2SO_3 is added to it; it is mixed, and after 3-4 min, submitted to polarography. The amount of DNP-amino acid is determined from the calibration graph. It can also be calculated from the formula $c = \frac{i_d}{Km^{2/3} t^{1/6}}$, using the value of K given in Table 2.

Corrections for decomposition in hydrolysis and for chromatographic loss are introduced in the same way as when other methods are used for the determination of DNP-amino acids, on the basis of control experiments with pure DNP-amino acids.

Di-DNP-histidine, in contrast to all the other DNP-amino acids, gives three waves in polarography (Fig. 1, polarogram 2). $E_{\frac{1}{2}}$ of the second and third waves almost agree with $E_{\frac{1}{2}}$ of the corresponding first and second waves of the other DNP-amino acids. $E_{\frac{1}{2}}$ of the first wave is more positive and at pH 8.2 equals -0.33 v. The height of this wave is, like the total diffusion current, proportional to the concentrations. This characteristic of the polarographic reduction of di-DNP-histidine permits the use of the method suggested by Wenger and others [10] for the determination of histidine in the form of di-DNP-histidine even in the presence of all the other amino acids. In this case, the amount of di-DNP-histidine can be determined by the height of the first wave. Since the first wave is not clearly distinguished from the second, best results are obtained, especially when there is considerable excess of other DNP-amino acids, by using differential polarography (Figs. 2 and 3). As Fig. 3 shows, the determination of di-DNP-histidine is possible in the presence of an 80-fold excess of the other DNP-amino acids.

SUMMARY

We have studied the polarographic behavior of DNP-amino acids. We have suggested a polarographic method for determining DNP-amino acids. We have established its usefulness for determining DNP-amino acids after their separation by paper chromatography.

We have shown the possibility of polarographic determination of di-DNP-histidine in the presence of the other DNP-amino acids.

In conclusion, we express our thanks to Prof. V. G. Klimenko for his constant advice and interest in our work.

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STABILITY TO HYDROLYSIS OF CERTAIN DI- AND TRIPEPTIDES CONTAINING L-PROLINE, L-HYDROXYPROLINE, AND GLYCINE

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Among the proteins collagen occupies a special position in that approximately 40% of its total amino acid composition is made up of glycine and the imino acids proline and oxyproline. On the basis of this it is suggested that in the collagen molecule there are successive units which contain glycine and the imino acids. Astbury [1] has suggested that such a unit is prolyl-glycyl-X, where X is an amino acid other than proline or glycine. Similar views have been held by Bergman [2]. Later studies of Neuman et al. [3] have shown, however, that imino acids, glycine, and other amino acids are present in approximately the ratio 2 : 3 : 4 – a circumstance which vitiates the hypothesis that the molecule consists of simple repeating units of the above-mentioned type. The studies of Grassman et al. [4] have shown that the imino acids are unevenly distributed in the molecule, being completely absent in some parts, while in other areas they are almost the only residues encountered besides glycine. A peptide has been obtained by enzymatic hydrolysis in the central portion of which there are 12 glycine residues, 10 proline residues, and 5 oxyproline residues in a total of 31 amino acids.

With the isolation of this fragment, it seemed to be established that there is no pattern in the distribution of imino acids in the collagen molecule. However, clear x-ray diffraction [5] and ultramicroscope studies [6] of this protein definitely indicate that a repeating fragment is present. The isolation by enzymatic hydrolysis of the two tripeptides glycyl-prolyl-oxyproline and glycyl-prolyl alanine in significant amounts [7] gives rise to the supposition that the repeating unit contains imino acids as an obligate component. In this connection it is of considerable interest to study the stability to hydrolysis, to enzymatic action, and to heat of the bonds between two imino acids and also those between an imino acid and those imino acids which are most frequently found in collagen, i.e., alanine, serine, and especially glycine.

This study is concerned with the stability to acid and alkaline hydrolysis of four dipeptides and five tripeptides which contain L-proline,* oxyproline, and glycine. The dipeptides glycyl-proline, prolyl-glycine, glycyl-oxyproline, and oxyprolyl-glycine were synthesized according to previously described methods [8]; the tripeptides prolyl-glycyl-proline, glycyl-prolyl-proline, glycyl-prolyl-oxyproline, and prolyl-glycyl-oxyproline were first synthesized in our laboratory [9]. Hydrolysis was carried out under conditions normally employed for collagen hydrolysis prior to the determination of the content of the di- and tripeptides obtained; it is thus possible to compare our results with those obtained previously on acid and alkaline hydrolysis of collagen [10, 11].

METHODS

Acid hydrolysis was carried out in an excess of 6N HCl at 37° for four days, and alkaline hydrolysis, in an excess of 1N NaOH under the same conditions. Hydrolysis products were identified by paper chromatography in a butanol – water – acetic acid (4: 5 : 1) system, occasionally in a phenol – water system. Chromatograms were sprayed with tolidine and isatin. Spraying with isatin makes it possible to distinguish the products not only by R_f, but also according to the color of the spots. This was of great help to us in a number of doubtful cases. Since it was the purpose of this study to determine the relative stability of the bonds, it was important to know not only the final composition of the hydrolysis mixture, but also the sequence of appearance of the various products. The latter requirement was met as follows: At intervals during hydrolysis samples were withdrawn and quickly place on dry ice; at the end of the hydrolysis period the samples were chromatographed together, thus enabling us to see the dynamics of the hydrolysis on a single chromatogram.

RESULTS

Acid Hydrolysis of Dipeptides

Glycyl-proline, prolyl-glycine, glycyl-oxyproline, and oxyprolyl-glycine were hydrolyzed. Three to four mg of chromatographically pure material was moistened with an excess (0.015-0.020 ml) of 6N HCl and left at 37°. Samples were withdrawn at 1 and 4 days after the beginning of hydrolysis. Hydrolyzates of glycyl-proline and prolyl-glycine were chromatographed in a butanol–

* The symbol "L" is omitted in further discussion.

TABLE 1. Chromatographic Characteristics of Peptide Hydrolysis Products

Peptides and amino acids	Color on isatin-sprayed chromatograms	R_f	
		Butanol – water—acetic acid (4:5:1)	Phenol – water
Glycine	Rose	0.12	0.41
Proline	Blue	0.33	—
Oxyproline	Pale sky blue	0.12	0.60
Glycyl-proline	Yellow	0.14	—
Prolyl-glycine	Blue	0.17	—
Glycyl-oxyproline	Reddish-brown	0.07	0.67
Oxyprolyl-glycine	Pale sky blue	0.13	0.60
Glycyl-prolyl-oxyproline	Rose	0.12	—
Prolyl-glycycl-oxyproline	Blue	0.12	—
Glycyl-prolyl-proline	Rose	0.32	—
Glycyl-prolyl-glycine	Yellow	0.13	—
Prolyl-glycyl-proline	Blue	0.33	—

Note: Glycine and all peptides with glycine on the amino end react with isatin to give pale spots.

water – acetic acid system and sprayed with tolidine.

Prolyl-glycine and glycyl-proline possess different stabilities. While glycyl-proline yielded very weak glycine and proline spots and a vivid dipeptide spot after one day of hydrolysis, prolyl-glycine yielded three spots of varying intensity corresponding to glycine, proline, and the dipeptide. Conclusions as to the relative amounts of amino acids on the basis of spot intensity are here completely valid since the peptides consist of the same amino acids.

Since the products of hydrolysis of glycyl-oxyproline and oxyprolyl-glycine have very similar R_f values in the butanol – water – acetic acid system (Table 1), we used a phenol – water system in which all the products, with the exception of oxyprolyl-glycine and oxyproline, are separated. In contrast to the first pair of dipeptides, glycyl-oxyproline and oxyprolyl-glycine respond in the same manner to acid hydrolysis. Oxyprolyl-glycine yielded two spots, which were identified as glycine and oxyproline mixed with oxyprolyl-glycine. Glycyl-oxyproline yielded three spots, indentified as glycine, oxyproline, and glycyl-oxyproline. R_f values of the products in the phenol – water system are given in Table 1.

Alkaline Hydrolysis of Dipeptides

Glycyl-proline, prolyl-glycine, glycyl-oxyproline, and oxyprolyl-glycine were subjected to alkaline hydrolysis. Chromatographically pure dipeptides [3-4 mg] were treated with an excess of 1N NaOH at 37°. Hydrolysate samples were withdrawn after 17 hours and four days.

As with acid hydrolysis, the glycyl-proline bond was noticeably more stable than the prolyl-glycine bond. After 17 hours of hydrolysis, prolyl-glycine yielded traces of proline and glycine and an intense dipeptide spot; after four days, the dipeptide spot had become hardly perceptible, while the glycine and proline spots had become much more intense. Glycyl-proline, after four days, yielded clearly visible spots, with the dipeptide spot the most intense. After 17 hours, glycyl-oxyproline yielded two spots, a very intense dipeptide spot and a very faint spot corresponding to glycine plus oxyproline. After four days, the same two spots were visible, but their relative intensities were just reversed (Fig. 1). The oxyprolyl-glycine hydrolyzate was chromatographed in a phenol – water system, and in general it yielded the same type of result as with acid hydrolysis.

Acid Hydrolysis of Tripeptides

Hydrolysis procedures were the same as for dipeptides. Samples were withdrawn after 1, 4, and 24 hours, and after 4 days.

Glycyl-prolyl-glycine. In the first three samples, three substances were identified on a tolidine-sprayed

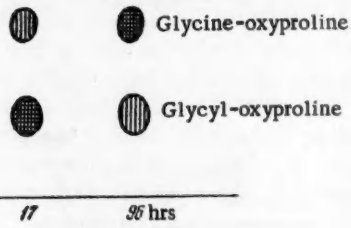

Fig. 1. Position of spots on a chromatogram of the acid hydrolyzate of glycyl-oxyproline.

chromatogram: glycine, prolyl-glycine, and glycyl-prolyl-glycine. After four days another weak spot corresponding in R_f to proline appeared. The four-day chromatogram was sprayed with isatin (Fig. 2); the absence of glycyl-proline was especially apparent.

Prolyl-glycyl-proline. After one hour's hydrolysis, two spots are visible; these correspond to prolyl-glycine and proline mixed with the tripeptide, which are not separated because of similar R_f values (Table 1). After 24 hours, four spots are visible: proline and the tripeptide, prolyl-glycine, glycyl-proline, and glycine. These were identified with the help of markers on a chromatogram sprayed with tolidine, and also by color on a chromatogram sprayed with isatin (Fig. 3).

Prolyl-glycyl-oxyproline. This tripeptide exhibited great stability to hydrolysis, being unchanged after one and four hours of treatment. After one day and four days, the changes were, on the whole, the same. Three spots, corresponding to proline, prolyl-glycine, and prolyl-glycyl-oxyproline in combination with glycine and oxyproline, were visible. Glycyl-proline was found neither on the chromatograms sprayed with isatin nor on those sprayed with tolidine.

Glycyl-prolyl-oxyproline. This tripeptide showed no marked changes in the course of one and four hours of hydrolysis. After one and four days, three spots of varying intensity, two strong ones and one pale one at the level of proline, were visible. The spot with an R_f of 0.12 (I) could be identified as glycine and tripeptide, and the spot with an R_f of 0.14 (II) could have been glycyl-proline; however, on the istain-sprayed chromatogram it was blue, a fact which vitiated this assumption.

To clarify the nature of spots (I) and (II), the hydrolyzate, in an amount containing about 50 micrograms of material, was applied in a line to chromatographic paper, and the chromatogram was developed for a day in a butanol – water – acetic acid system. The chromatogram was then dried, and two strips were cut from each side and sprayed with isatin in order to localize the spots.

Strips at R_f 0.12 and R_f 0.14 were then cut out and eluted with hot water (two 10-ml portions). The water was evaporated under a vacuum and both eluates were boiled six hours in 6N HCl on a water bath. At the completion of hydrolysis, the excess HCl was distilled off and the material chromatographed. A chromatogram of the hydrolyzate of the spot with an R_f of 0.14, which was sprayed with tolidine, gave two spots indentified with the aid of markers as proline and oxyproline. Treatment of the same chromatogram with isatin revealed a blue and a sky-blue spot, and thus the material in question is prolyl-oxyproline.

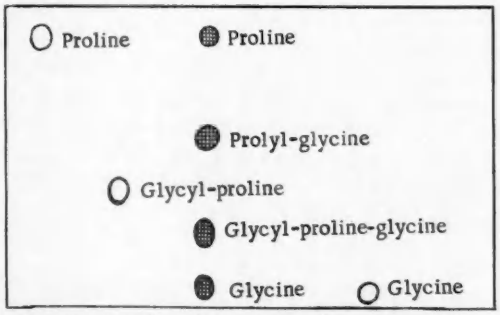

Fig. 2. Position of spots on a chromatogram of the acid hydrolyzate of glycyl-prolyl-glycine.

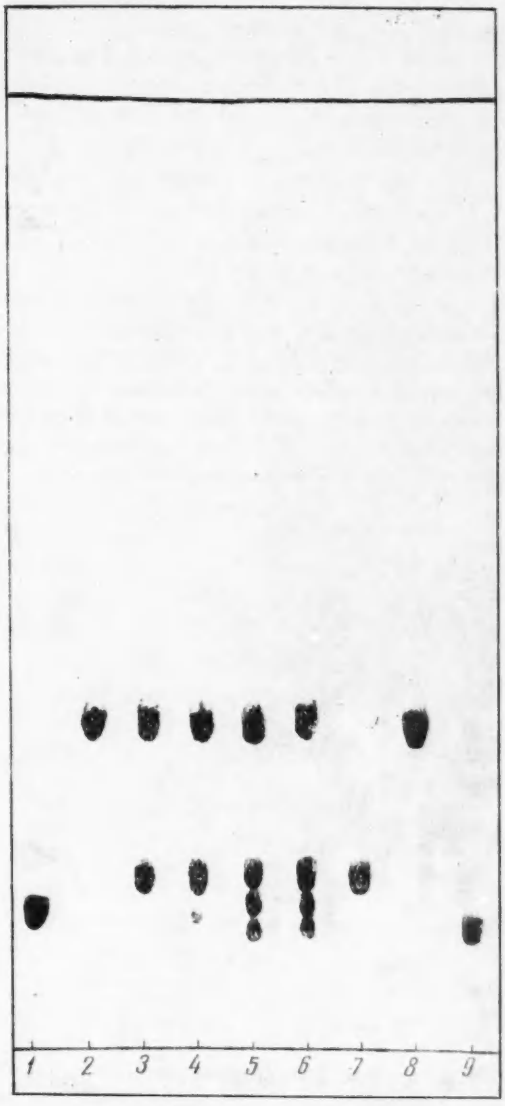

Fig. 3. Chromatogram of the acid hydrolyzate of prolyl-glycyl-proline. Solvent system: butanol – water – acetic acid (4: 5 : 1). Chromatogram was sprayed with tolidine. Markers: 1) glycyl-proline; 2) proline; 7) prolyl-glycine; 8) prolyl-glycyl-proline; 9) glycine. Duration of hydrolysis in hours: 3) 1; 4) 4; 5) 25; 6) 96.

Proline was found in the hydrolyzate of the spot of R_f 0.12, a circumstance which testifies to the fact that after four days' hydrolysis a considerable portion of the tripeptide is still unhydrolyzed. After six days, the spot of R_f 0.14, which is ordinarily blue on an isatin-sprayed chromatogram, shows a green tinge, which suggests the formation of glycyl-proline.

Glycyl-prolyl-proline. This is the most stable of all the tripeptides studied – it shows no noticeable change after 24 hours' hydrolysis. After four days, two clear spots corresponding in R_f to glycine and a mixture of proline with the tripeptide are visible on a tolidine-sprayed chromatogram. Neither on tolidine-sprayed nor on isatin-sprayed chromatograms was glycyl-proline found.

Alkaline Hydrolysis of Tripeptides

Hydrolysis procedures were the same as those employed with dipeptides; samples were taken at 1, 4, and 24 hours, and 4 days.

Glycyl-prolyl-proline. After one hour of hydrolysis, two spots were found: glycyl-proline and proline mixed with the tripeptide. After four hours, a faint glycine spot appeared which became markedly more intense after one day of hydrolysis. After four days, the same three spots were visible. It is interesting to note that in the first two samples glycine was not found.

Prolyl-glycyl-proline. After one hour, four spots become visible: glycine, glycyl-proline, prolyl-glycine, and proline mixed with the tripeptide. After four days, the same four spots are visible, but they have gradually become more intense. As with acid hydrolysis, both glycyl-proline and prolyl-glycine are present.

Prolyl-glycyl-oxyproline. During the hydrolysis of this compound and of glycyl-prolyl-oxyproline, samples were taken at 17 hours and four days. As early as 17 hours, four spots, identified with markers as glycyl-oxyproline, the tripeptide in combination with oxyproline and glycine, prolyl-glycine, and proline are visible. The same identifications were made after treatment of the chromatogram with isatin. With this treatment, glycyl-oxyproline showed up as a weak rust-colored spot.

Glycyl-prolyl-oxyproline. After 17 hours' hydrolysis two spots corresponding to the tripeptide mixed with oxyproline and glycine and to glycyl-proline were found. After four days, a proline spot appeared. On an isatin-sprayed chromatogram, the first spot was rose-colored with a sky-blue tinge, which can be due only to oxyproline. The second spot was pale yellow, which is typical of glycyl-proline.

Glycyl-prolyl-glycine. Samples were taken after 1, 4 and 24 hours, and after 4 days. Beginning with the 4-hour sample, the following substances were found: glycyl-proline, prolyl-glycine, proline, and glycine. Spots were identified with markers and by their color reactions with isatin.

DISCUSSION

It should be pointed out that the results described are qualitative in nature. Even though the methods used precluded a strict quantitative evaluation of the stability of one bond or another, they did enable us to establish relative bond stabilities. From this it becomes clear that we obtained the more clear-cut results with tripeptides, in which there are always two different types of bond. With dipeptides, in which it is necessary to estimate the extent of hydrolysis, we obtained less clear-cut results. Actually, no substantial difference between oxyprolyl-glycine and glycyl-oxyproline in response to acid or alkaline hydrolysis was brought out. However, qualitative observations clearly showed that glycyl-proline is more stable to hydrolysis than prolyl-glycine.

The small specificity of alkaline hydrolysis with respect to the rupture of the peptide bond of prolyl-glycine and glycyl-proline should be noted. In most cases, alkaline hydrolyzates contained prolyl-glycine, glycyl-proline, and glycyl-oxyproline, a fact which indicates equal ease of rupture of all possible bonds.

Acid hydrolysis showed greater specificity. Of four possible modes of formation of glycyl-proline (decomposition of glycyl-prolyl-glycine, of prolyl-glycyl-proline, of glycyl-prolyl-oxyproline, or of glycyl-prolyl-proline) only the second yielded this peptide in perceptible amounts, though to a smaller extent than prolyl-glycine.† In all five tripeptides, the glycyl-imino acid bond was preferentially attacked (Table 2). From the course of decomposition of prolyl-glycyl-proline it appears that although the prolyl-glycine bond is decomposed more slowly than the glycyl-proline bond, the difference between them is slight. It is interesting to note that during acid hydrolysis of tripeptides the prolyl-oxyproline bond possesses great stability. The stability of the peptide bond between two imino acids, which is confirmed in this study, becomes more and more apparent. Thus, the prolyl-proline sequence is found in the melanophore hormone and in the peptide glycyl-prolyl-oxyproline, which was isolated by Schrohenloner from collagen. We had pointed out still earlier the great stability of the prolyl-proline bond as compared to the prolyl-glycine bond with respect to thermal destruction of prolyl-prolyl-glycine [13]. From the foregoing it should be concluded that one cannot speak of peptide bond stability in general, since the picture is entirely different with a dipeptide than with a tripeptide. In dipeptides, the prolyl-glycine bond is less stable to acid hydrolysis, while in tripeptides the glycyl-proline bond is least stable.

† In this connection, it should be mentioned that the anhydride of prolyl-glycine forms prolyl-glycine in acid medium and glycyl-proline in alkaline medium [12].

TABLE 2. Products of Acid Hydrolysis of Tripeptides

Peptides hydrolyzed	Duration of hydrolysis			
	1 hr	4 hr	24 hr	4 days
Glycyl-prolyl-proline	Glycyl-prolyl-proline			Glycine, proline, glycyl-prolyl-proline. Possibly prolyl-proline
Prolyl-glycyl-proline	Prolyl-glycine, proline, prolyl-glycyl-proline		Proline, glycine, prolyl-glycine, glycyl proline, prolyl-glycyl-proline	
Prolyl-glycyl-oxyproline			Proline, prolyl-glycine, the tripeptide mixed with glycine and oxyproline	
Glycyl-prolyl-oxyproline	Glycyl-prolyl-oxyproline		Proline, glycine, prolyl-oxyproline, glycyl prolyl-oxyproline	
Glycyl-prolyl-glycine	Glycine, proline, glycyl-prolyl-glycine, prolyl-glycine			Glycine, proline, glycyl-prolyl-glycine, prolyl-glycine

SUMMARY

Prolyl-glycine is less stable to acid or alkaline hydrolysis than glycyl-proline. The prolyl-oxyproline bond was more stable than the glycyl-proline bond during acid hydrolysis of glycyl-prolyl-oxyproline. It was shown that during acid hydrolysis of the tripeptides studied the glycine-imino acid bond is the first to be attacked. Acid hydrolysis of tripeptides was shown to be more specific than alkaline hydrolysis.

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THE SYNTHESIS OF THE β-AMIDE OF α-KETOSUCCINIC (-4-C^{14}) ACID

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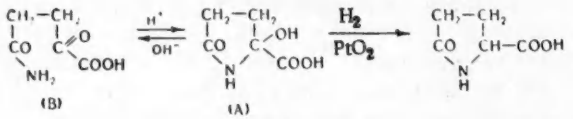
Mardashev and Semina [1] in experiments with glyoxylic acid, and Meister and Tice [2, 3] in experiments with α-ketoacids showed that glutamine and asparagine react by an enzymatic transamination:

$$RCOCOOH + H_2NCO(CH_2)_nCH(NH_2)COOH \longrightarrow$$

$$\longrightarrow RCH(NH_2)COOH + H_2NCO(CH_2)_nCOCOOH \quad (I),$$

where n = 1, 2.

According to the data from the laboratory of Meister [3, 4], glutamine and asparagine are transformed by this reaction into the corresponding ω-amide of the α-keto-acid (I). The synthesis of these ω-amides, especially with labeled C^{14}, is of value for the study of glutamine and asparagine metabolism. The ω-amides of α-ketoglutaric and α-ketosuccinic acids were obtained by Meister by oxidation of the corresponding ω-amides of the dicarboxylic amino acids with the L-amino acid oxidase of snake venom in the presence of catalase; in the absence of catalase the H_2O_2 which is formed causes decarboxylation. Depending on the experimental conditions, both compounds can be isolated in one of two forms, A and B [5]. Form B has all the properties of a ketoamide: It forms a 2, 4-dinitrophenylhydrazone, it is deaminated by ω-amidase of the liver, it is quantitatively decarboxylated by $Ce(SO_4)_2$, and also by hydrogen peroxide (H_2O_2). Form A does not react with 2, 4-dinitrophenylhydrazine and is not decarboxylated by H_2O_2. In an alkaline medium form A goes over to form B. Recently Otani and Meister [6] showed that the A form of the γ-amide of α-ketoglutaric acid has a cyclic structure and is easily hydrogeneated to 5-oxopyrrolidine-2-carboxylic acid:



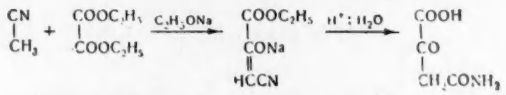
The B form of the β-amide of α-ketosuccinic acid, which has an open chain, is sufficiently stable, and when the medium is acidified slowly enough, is transformed into the A form; the reverse change in an alkaline medium occurs rapidly. The structure of the A form of the β-amide of α-ketosuccinic acid is still not completely established; it follows from the determination of the molecular weight that the A form is a dimer [6].

In the present study we describe the chemical synthesis of the β-amide of α-ketosuccinic acid by a method which is also suitable for obtaining a compound labeled with C^{14}.

In the hydrolysis of the diamide of oxaloacetic acid under conditions analogous to those for the synthesis of the β-amide of malic acid [7] there was obtained a monoamide which, however, differed in melting point from the compound (I) (n = 1), and which is evidently a structural isomer of the latter (α-amide of α-keto-succinic acid).

The compound (I) (n = 1) was obtained by us as the result of hydrolysis of the product of ester condensation of acetonitrile with oxalic ester. The condensation of oxalic ester with acetonitrile was first carried out in 1888 [8], and then was repeated under different conditions [9, 10]. However, the final product, β-cyanopyruvate, has not previously been isolated in the free form,* but only in the form of a sodium derivative.†

Therefore we carried out the hydrolysis of the Na derivative of the condensation product:



Attempts to carry out the hydrolysis in ether saturated with HCl with later addition of the theoretical amount of H_2O [13] or an alkaline solution of H_2O_2 [14] were unsuccessful. Good results were obtained only by hydrolysis with concentrated HCl in the cold [15]. Splitting out of Na, hydrolysis of the ester group, and saponification of the nitrile group to the amide were carried out in one step. The hydrolysis lasted 20-25 days at 0-5°. The resulting

* We also could not obtain it in the condensation of CuCN with ethyl β-bromopyruvic acid.

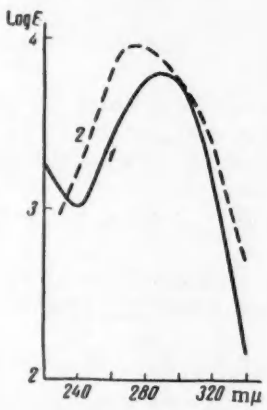
† The ethyl β-alkyl-β-cyanopyruvates have been obtained in the free form.

substance was sufficiently pure and the yield was satisfactory (30-35%). The β-amide of α-ketosuccinic acid was characterized by melting point, elementary analysis, as the 2, 4-dinitrophenylhydrazone, by the ultraviolet spectrum (Figure), by paper chromatography, and also by conversion to asparagine by enzymatic transamination with α-amino acids. The substance which we obtained was quantitatively decarboxylated by $Ce(SO_4)_2$, but not by H_2O_2, which confirms the identity of the compound which we synthesized with the A form of the β-amide of α-ketosuccinic acid. The labeled β-amide of α-ketosuccinic acid $H_2NC^{14}OCH_2COCOOH$ was obtained in one step, starting from acetonitrile (-1-C^{14}); The latter was obtained by the method of Walden [16] from $KC^{14}N$ and dimethyl sulfate.

According to the data of Cornish and co-workers [17] under these conditions there is formed an azeotropic mixture of acetonitrile and water which boils at 76° and contains 15% water. After removal of the water the dry ether solution of acetonitrile (-1-C^{14}) was used for condensation.

EXPERIMENTAL

Na-derivative of ethyl β-cyanopyruvic acid. To a suspension of C_2H_5ONa (obtained from 28 g of Na and 46 g of ethyl alcohol) in 400 ml of absolute ether, 146 g of diethyl oxalate was added rapidly with stirring. To the gently boiling solution, 41 g of dry acetonitrile was added slowly by drops, and the mixture was boiled for one hour; then it stood for 12-15 hours at room temperature, and was then evaporated to dryness. The residue was suspended in 400 ml of absolute ether and filtered, washed with absolute ether, and dried in a vacuum. Yield, 153 g (95%).



Ultraviolet absorption spectrum of the β-amide and the β-ethyl ester of α-ketosuccinic acid in 0.1 N NaOH. 1) β-amide of α-ketosuccinic acid ($6.48 \cdot 10^{-5}$ M); 2) β-ethyl ester of α-ketosuccinic acid ($6.85 \cdot 10^{-5}$ M).

β-amide of α-ketosuccinic acid. We added 32.6 g (0.2 M) of well ground Na derivative of the ethyl ester of β-cyanopyruvic acid in small portions to 120 ml of concentrated HCl in such a way that the temperature of the reaction mixture did not exceed 20-25°, and allowed it to stand 15-20 min at this temperature; then it was filtered from the precipitate of NaCl and the latter was washed with 10 ml of cold, concentrated HCl. The filtrate was combined with the wash liquid and kept 20-25 days at 0-5°. The white, finely crystalline precipitate was filtered off and washed successively with concentrated HCl absolute alcohol, and absolute ether. It was dried in a vacuum over KOH; yield, 7.9 g (30%).‡ M. p. 140° (uncorrected). The water solution of the substance gave a positive reaction with a 1% $FeCl_3$ solution.

Analysis: Found % : C 37.29; H 3.87; N 10.59
Calculated % : C 36.65; H 3.85; N 10.68

The substance was quantitavely decarboxylated by $Ce(SO_4)_2$, but not by H_2O_2. Chromatographic study was carried out in 80% phenol (I) and in a mixture of n-butanol-formic acid-water (60 : 20 : 8) (II) on Whatman No. 4 paper. The chromatogram was developed by the method of Wieland [18]. The substance gave one spot which in the ultraviolet fluoresced with a yellow-green color; R_f (I) 0.35, R_f (II) 0.25. The standard β-amide of α-ketosuccinic acid•• had the same R_f on the chromatogram as the substance which we synthesized. The ultraviolet spectrum of the substance (Figure) had the characteristic absorption band at 290 mμ which was identical with that given in the literature for the β-amide of α-ketosuccinic acid [5].

2,4-Dinitrophenylhydrazone of the β-amide of α-ketosuccinic acid. We dissolved 260 mg (0.002 moles) of the β-amide of α-ketosuccinic acid in 50 ml of 0.2 N NaOH and allowed it to stand for ten min; then we added 75 ml of a warm 1% solution of 2, 4-dinitrophenylhydrazine in 2 N HCl. We cooled it and extracted three times with 200 ml of ethyl acetate. The resulting ethyl acetate solution was extracted five times with 100 ml of 10% Na_2CO_3 solution and the ethyl acetate was discarded. The extract was washed twice with 250 ml of ethyl acetate and the wash liquid was discarded. We acidified with concentrated HCl and extracted three times with 250 ml of ethyl acetate. The ethyl acetate extracts were combined, dried over Na_2SO_4, and evaporated to dryness. Yield 95 mg. M. p. 181-182° (corrected) from ethyl acetate (the literature gives 183° [19]). Chromatography in phenol saturated with a 10% solution of Na_2CO_3 on

‡ The substance could be recrystallized from hot water.

•• This reagent for the identification was kindly sent to us by A. Meister, to whom we express our thanks.

Whatman No. 4 paper gave one spot with R_f 0.65 (the literature gives R_f 0.62 [19]).

Acetonitrile (-1-C^{14}). In a 50 ml round bottom flask we placed 0.05 M $KC^{14}N$ and 4 ml of H_2O and then with strong cooling, 4.5 ml of freshly distilled dimethyl sulfate (87-88°, 22 mm Hg). The flask was connected with a condenser with downward cooling and the apparatus was placed in a cooling mixture. Several minutes after removal of ice a stormy reaction began, the course of which was controlled by an ice bath. After 15-20 min the reaction was complete; the acetonitrile (-1-C^{14}) was distilled on an oil bath at 120-140° and the 76-80° fraction was collected and dried 48 hours over Na_2SO_4, then dissolved in absolute ether (1:1) and dried over P_2O_5. The yield was 90% calculated on the $KC^{14}N$. The dry ether solution was used for the condensation which occurred without difficulty with a yield of 95% based on acetonitrile (-1-C^{14}).

β-amide of α-ketosuccinic acid (-4-C^{14}). The β-amide of α-ketosuccinic acid, labeled on the fourth carbon atom, was obtained as described above for the model synthesis of unlabeled substance.

Diamide of α-ketosuccinic acid. The diamide of α-ketosuccinic acid was obtained by the method of Thomas-Mamert [20] from chlorofumaric ester which was obtained according to Claus [21]. M. p. 180° (the literature gives 180° [20]).

Semiamide of α-ketosuccinic acid. We suspended 3.25 g of $Ba(OH)_2 \cdot 8H_2O$ (0.01 mole) in 40 ml of water and added 2.6 g of the diamide of α-ketosuccinic acid. The mixture took on a green-yellow color and almost everything dissolved. It was placed in a thermostat at 48-50° for 1-1.5 hours. After it had cooled to room temperature, a crystalline precipitate of barium salt appeared. The precipitate was filtered off, washed with cold water, and dried in a vacuum. Yield 2.2 g (55%). It was suspended in water and the barium was precipitated with 5 N H_2SO_4. The solution was decolorized with Norit and evaporated until crystallization began. The residual mother liquor was sucked off and the crystals were washed with cold water, alcohol, and ether. Yield 0.7 g (47%). M. p. 159-160° (uncorrected). The substance gave a positive reaction with $FeCl_3$.

Analysis: Found %: N 10.60
Calculated %: N 10.68

SUMMARY

We have presented a method for the chemical synthesis of the β-amide of α-ketosuccinic acid. On the basis of this method we have carried out the synthesis of the β-amide of α-ketosuccinic acid (-4-C^{14}).

The authors use this opportunity to thank V. I. Maimind and K. M. Ermolaev for much valuable advice during this work.

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ON THE FATE OF FRUCTOSE-1,6-DIPHOSPHATE AND FRUCTOSE-6-PHOSPHATE ADMINISTERED TO ANIMALS

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In dealing with the problem of controlling metabolism with a view to controlling the functioning of an organism, the use of biostimulators such as coenzymes and hormones should be augmented by the use of metabolites and antimetabolites. A metabolite such as fructose-1, 6-diphosphate (F-1, 6-DP) has already found use in clinical practice as an anti-shock agent [1, 2]. However, at the present time the fate of F-1, 6-DP administered to an animal is little known, although it is of great theoretical and practical interest.

In recent years there have been a number of studies of this question. Natelson et al. [3], after peroral administration of the calcium salt of F-1, 6-DP to humans, observed an increase in inorganic phosphorus, and especially in organic phosphorus, of the blood plasma. Lucareli [4] observed that after intravenous administration of the sodium salt of F-1, 6-DP to dogs (14 mg per kg animal weight) there is a sharp rise in the diphosphate content of the blood, which returns to normal in 30 minutes. When F-1, 6-DP was administered intravenously to rats (100 mg/kg), neither fructose nor organic phosphorus was found in the urine. On this basis the author concludes that the F-1, 6-DP is completely utilized However, in the experiments of Taugner et al. [5], who investigated the excretion of phosphorus by dog kidneys, intravenously administered disodium F-1, 6-DP was partially excreted in the urine.

Pellegrini and Pedrazzi [6] showed that with the intravenous administration of 1 g (evidently ~20 mg/kg) of the sodium salt of F-1, 6-DP to healthy humans there is a slight increase of reducing substances in the blood (with a subsequent return to normal) which is accompanied by a decrease in pyruvic and lactic acids. Finally, Poloza et al. [7] observed that after the ingestion per os of 50 g of the sodium salt of F-1, 6-DP by humans the blood sugar level remains unchanged and there is an increase in the concentration of P_{inorg} and pyruvic acid which is accompanied by a decrease in lactic acid. The data presented in the foregoing studies are comparable only to a very small extent, inasmuch as different preparations (calcium and sodium salts) were used at different dose levels and were administered in various ways, test animals were different, and various types of determinations were made.

In a few studies in which levels of metabolites other than F-1, 6-DP in the blood were determined [6, 7], attention was devoted chiefly to the final product of glycolysis, lactic acid, on the assumption, apparently, that glycolysis represents the main pathway of F-1, 6-DP utilization. Data obtained in such studies, however, reveal a decrease in lactic acid after administration of F-1, 6-DP and indicate rather that the glycolytic degradation of this compound is not the main pathway of its utilization. Thus, the chief mode of F-1, 6-DP utilization is still obscure.

The principal objective of this study was to elucidate the major pathway of F-1, 6-DP utilization in vivo. In the course of the study we investigated the content in the blood not only of the final products of glycolysis but also of certain intermediates, the phosphotrioses. Moreover, it seemed necessary to explore the possibility of a hydrolytic breakdown, and we therefore determined fructose-6-phosphate (F-6-P) and fructose levels in the blood. In addition special experiments were set up in which F-6-P was administered intravenously. The urine was also examined. Where necessary, in vivo experiments were supplemented by in vitro experiments on the blood.

METHOD

Preparations. Sterile 10% solutions of the sodium salt of F-1, 6-DP prepared according to Stepanenko and Bobrova [8, 9] were used. The barium salt of F-6-P was prepared by careful hydrolysis of F-1, 6-DP according to Neuberg et al. [10] and was converted to the sodium salt with Na_2SO_4. The solution of the sodium salt was adjusted to pH 7.3-7.5 with 2N HCl, sterilized by passage through a bacterial filter, and distributed among sterile ampules.

Experimental procedure with animals. Experiments were performed on rabbits. Sterile 10% solutions of the sodium salts of F-1, 6-DP and F-6-P were injected into the auricular vein in almost equivalent quantities, 0.5 and 0.4 g/kg respectively, in a period of approximately 1-1.5 minutes. Just before injection and at various

intervals after injection, which are indicated on the tables and figures, 1-ml samples of blood were withdrawn and mixed with 3 ml of 8% TCA. The precipitated protein was centrifuged off, and samples were withdrawn from the supernatant for determination of: P_{inorg} by the Fiske-SubbaRow method, F-1, 6-DP and F-6-P according to Roe et al. [11, 12], phosphotrioses according to Meshkova and Severin [13], and lactic acid according to Barker and Summerson [14]. Reducing substances were determined according to Hagedian and Jensen.

In some experiments, urine excreted over a 24-hour period was analyzed by the method described above, and also by the chromatographic method, for phosphoric acid esters of fructose, and free fructose.

Experiments in vitro. Three volumes of citrated blood of rabbits (temp. 37°) was mixed with one volume of a F-1, 6-DP or a F-6-P solution in concentration of 5, 2.5, and 1.25% (heated to 37°). The mixture was incubated one hour at 37°. At the beginning of the experiment and after 30 and 60 minutes samples were withdrawn for analysis of P_{inorg} and fructose.

Chromatographic methods. When necessary, F-1, 6-DP, F-6-P, and free fructose were identified chromatographically. For blood analysis, TCA filtrates free of protein were used. The filtrate was extracted with ether, neutralized with 2N NaOH to pH 7.4-7.6, and chromatographed on Leningrad paper which was then treated with diphenylamine according to a method described earlier [15].

For urine analysis, whole urine was applied to chromatographic paper in amounts from 0.01 to 0.2 ml and separation was carried out (as with blood) in an n-butanol-acetic acid-water (74 : 19 : 50) system [16]. The chromatogram was then sprayed with diphenylamine.

TABLE 1. Changes in the P_{inorg} content of blood (in mg%) after administration of F-1,6-DP.

Time of sample withdrawal, min.	Expt. number			
	1	2	3	4
Before admin.	4,0	5,0	2,7	2,1
After 3	18,0	16,0	14,8	10,3
" 15	11,4	13,4	11,7	7,7
" 30	6,8	14,8	13,5	10,3
" 45	9,2	13,8	13,0	9,5
" 60	7,8	—	10,9	7,7
" 120	6,2	—	6,9	6,4
" 180	5,0	—	3,3	4,5

RESULTS

Changes in inorganic phosphorus content of the blood after administration of fructose-1,6-diphosphate. As Table 1 shows, there is a rapid rise in P_{inorg} as early as the first 3 minutes after administration of F-1, 6-DP. During the next 10-15 minutes (less frequently, 30 minutes), there is as a rule a certain decrease in phosphorus content and this is followed by a second rise to a maximum at 30-45 minutes from the beginning of the experiment.

During the next 30-45 minutes the phosphate content gradually falls, and after three hours it has approached the normal level. Being at first doubtful that the double rise actually occurs, we set up a supplementary series

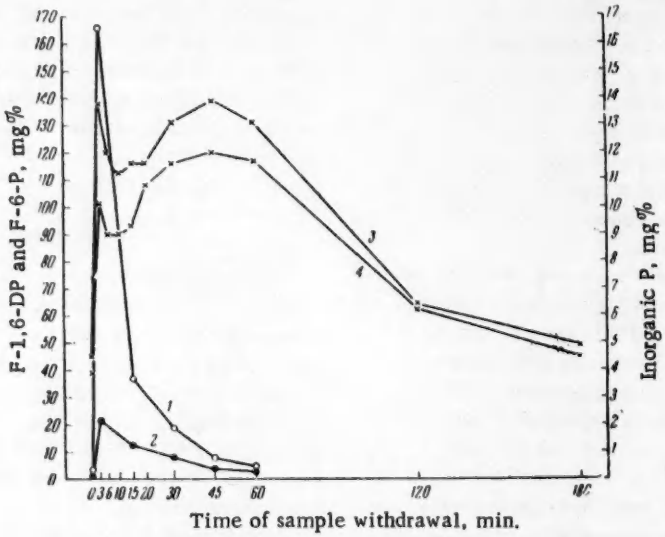


Fig. 1. Typical curves showing changes in F-1,6-DP, F-6-P, and P_{inorg} contents of the blood after administration of F-1,6-DP. 1) F-1,6-DP; 2) F-6-P; 3 and 4) P_{inorg} (corresponding to experiments 5 and 6).

of experiments in which 0.2 ml samples were withdrawn at smaller intervals, and the same results were obtained (Fig. 1)

The cause of the pattern observed may be a stepwise hydrolysis of F-1,6-DP consisting of a rapid hydrolysis to F-6-P, and then, perhaps, a slow hydrolysis of the latter. To test this hypothesis, F-6-P was administered to animals and its hydrolysis was studied.

Changes in inorganic phosphorus content of blood after administration of fructose-6-phosphate. The curves for changes in inorganic phosphorus of the blood are completely different when F-6-P is given as compared with F-1,6-DP (Fig. 2). Here there is a gradual rise in phosphate to a maximum at 30-45 minutes. This si followed by a gradual decline, with the normal level being reached after about 3 hours. A comparison of the curves for F-1,6-DP and F-6-P (Figs. 1 and 2) yields interesting results. In the first few minutes, during which there is a large increase in P_{inorg} with administration of F-1,6-DP, there is only a very slight increase with F-6-P. Of special interest is the coincidence of the second rise of P_{inorg} after F-1,6-DP administration with the rise to a maximum following F-6-P administration (at 30-45 minutes after the beginning of the experiment). These data therefore support the assumption of a two-step degradation of F-1,6-DP, the first step being a rapid hydrolysis of phosphate from C_1 and the second a comparatively slow hydrolysis of the second phosphate residue from F-6-P or the products of its further conversion.

The increase in P_{inorg} of the blood which occurs during the first few minutes after the administration of F-1,6-DP and of F-6-P may be used to estimate the extent of hydrolysis of these phosphates. This is possible because of the extremely slow hydrolysis of phosphate from F-6-P as compared with F-1,6-DP (judging by the very slow rise in P_{inorg}) (Fig. 2). In our calculations we assumed that F-1,6-DP is hydrolyzed in the first few minutes to F-6-P and that the volume of blood is on the average 85 ml/kg animal weight. These calculations are only approximate (since phosphate of the blood passes into other tissues in part and is excreted in the urine in part), and their results, presented in Table 2, are probably somewhat low.

From Table 2 it follows that in the first 3 minutes more than 1/4 of the F-1,6-DP administered is hydrolyzed (taking into consideration simultaneous excretion); hydrolysis of F-6-P is more than an order of magnitude slower.

Although a study of changes in P_{inorg} content of the blood provides a basis for certain conclusions, direct determination of fructose phosphates is the most important approach to the problem.

Results of fractionation and determination of fructose phosphates in blood after their administration. In the method of fractionation of F-1,6-DP and F-6-P which was used [12], precipitation of fructose esters was carried out in phosphate buffer at pH 8.3. This method is superior to that of Umbreit [17] in which, because of the high solubility of F-1,6-DP, only 60-80% is precipitated and the F-6-P fraction is strongly contaminated [18, 19]. Results are presented in Tables 3 and 4.

As seen in Table 3 and Fig. 1, the contents of both F-1,6-DP and F-6-P in the blood fall rapidly, and by an hour after administration of F-1,6-DP they have reached normal levels, which testifies to a very rapid utilization (excretion) of these compounds. A striking feature of this experiment is the extremely rapid conversion of diphosphate to monophosphate, the content of which is ~30% that of the diphosphate 3 minutes after the beginning of the experiment (converted to equivalents of fructose). This value

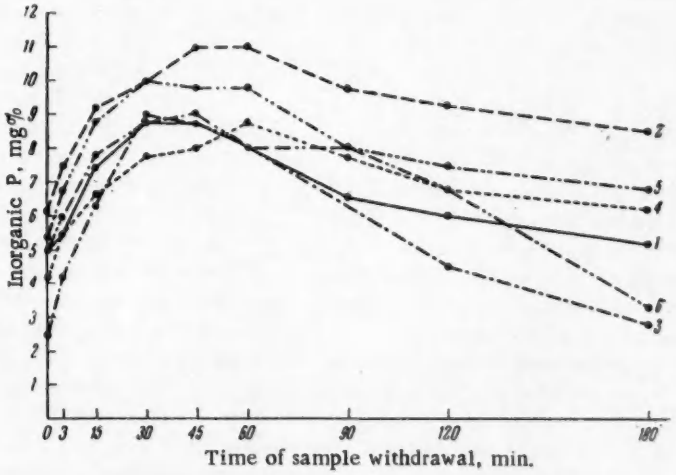


Fig. 2. Curves showing changes in P_{inorg} content of blood after administration of F-6-P. Figures on the curve indicate experiment numbers.

TABLE 2. Extent of Hydrolysis of Fructose Phosphates during the First Three Minutes after Administration (according to rough calculations)

Amt. of fructose phosphate administered, g	Increase in $P_{inorg.}$, mg%	Fructose phosphate hydrolyzed		Amt. of fructose phosphate administered g	Increase in $P_{inorg.}$, mg%	Fructose phosphate hydrolyzed	
		g	% of amt. administered			g	% of amt. administered
Fructose-1,6-diphosphate				Fructose-6-phosphate			
1,50	6,9	0,240	15,3	1,20	1,40	0,035	2,9
1,50	14,0	0,492	32,8	1,10	1,25	0,029	2,6
1,175	11,0	0,302	25,7	1,24	0,50	0,013	1,0
1,50	12,15	0,420	28,0	1,20	1,25	0,031	2,6
1,65	8,21	0,316	19,1	1,12	1,00	0,024	2,1
Average			24,2	Average			2,2

TABLE 3. Changes in Contents of F-1,6-DP and F-6-P in the Blood (Mg%) after Administration of F-1,6-DP

Time of sample withdrawal, min.	F-1,6-DP				F-6-P			
	Expt. number							
	1	2	3	4	1	2	3	4
Before administration	3,3	1,90	2,37	3,32	0,0	0,0	1,01	2,37
After 3	246,48	163,06	165,66	127,27	54,08	27,72	21,97	32,79
" 15	46,93	—	36,97	46,22	15,21	13,18	12,51	18,93
" 30	15,64	17,54	19,43	23,70	8,11	9,46	8,11	13,86
" 45	8,13	7,11	8,13	9,48	2,37	3,72	4,39	8,11
" 60	5,21	—	5,21	6,16	1,35	—	3,04	4,39

Note. In this and succeeding tables, amounts of F-1,6-DP and F-6-P are calculated by multiplying experimentally determined values by 2.37 and 1.69, respectively, to convert to equivalent amounts of the sodium salts.

is in good agreement with calculated values (Table 2), according to which more than a quarter of the F-1,6-DP administered is hydrolyzed in the first 3 minutes.

The data of Table 4 and Fig. 3 indicate that administered F-6-P also disappears from the blood stream very rapidly, in 30-60 minutes.

Chromatographic separation of fructose-1,6-diphosphate, fructose-6-phosphate, and fructose of the blood. Three minutes after administration of F-1,6-DP, F-6-P was found in the blood, but free fructose was not found. The absence of fructose suggests that hydrolysis of F-1,6-DP to F-6-P does not proceed further, or only to a slight extent. The organism evidently utilizes F-6-P, and fructose, which is excreted by the kidneys with comparative ease, is not formed or is formed to only a slight extent.

Changes in content of reducing substances in the blood after administration of fructose phosphates. Data on changes in the level of reducing substances in the blood following administration of fructose phosphates are presented in Table 5.

As the data of Table 5 show, hyperglycemia appearing after administration of fructose phosphates is in general very slight. The maximum increase of reducing substances over the original levels is 5-23%, and only in rare cases is it greater than 30%. In at least half the experiments, the maximum increase in reducing substances is observed not immediately after administration but after 15-45 minutes. As is well known, the reducing capacities of F-1,6-DP and F-6-P are 40.6 and 79% that of glucose, respectively. The gradual increase in reducing strength of the blood observed in a number of experiments evidently depends on the conversion of fructose esters into materials possessing a greater reducing capacity, which may be F-6-P, G-6-P, glucose, or phosphotrioses. The different rates of formation and utilization of these compounds, which depend on individual peculiarities of the animals, may be the cause of variability in results obtained from experiment to experiment.

Changes in phosphotriose content of blood after administration of fructose-1,6-diphosphate. As already indicated, more than 1/4 of the F-1,6-DP is hydrolyzed in the first three minutes after administration. This testifies to the fact that at least the major part of the F-1,6-DP is first converted to F-6-P. The possibility that glycolysis occurs

TABLE 4. Changes in F-6-P Content of Blood (Mg%) after Administration of F-6-P

Time of sample withdrawal, min.	Expt. number				
	1	2	3	4	5
Before administration	5,07	3,04	3,04	3,04	6,76
After 3	76,05	67,94	87,20	87,20	61,68
" 15	20,28	16,22	27,72	25,35	21,97
" 30	12,67	7,44	12,51	8,79	15,21
" 45	5,07	4,39	6,08	5,07	—
" 60	5,07	3,04	5,07	3,04	7,60
" 90	5,07	3,04	3,04	2,37	6,76
" 120	7,60	3,04	3,04	2,37	7,60

TABLE 5. Changes in the Content of Reducing Substances in the Blood (Mg% of Glucose) after Administration of F-1,6-DP and F-6-P

Time of sample withdrawal, min.	Expts. with F-1,6-DP						Expts. with F-6-P			
	1	2	3	4	5	6	1	2	3	4
Before administration	133	100	105	113	123	80	147	141	126	127
After 3	—	—	—	—	—	105	174	152	153	133
" 15	128	111	98	127	138	98	159	134	164	130
" 30	124	106	119	121	131	104	150	159	148	131
" 45	141	95	129	121	121	107	156	186	126	124
" 60	133	95	129	123	130	111	147	186	128	124
" 120	128	95	118	125	131	111	132	143	130	121
" 180	122	94	109	108	127	110	121	132	138	108
Maximum increase over original level, %	6	11	23	12	12	38	20	32	22	5

could not be excluded, however, and we therefore considered it necessary to determine whether the phosphotriose levels in the blood change (Table 6).

As Table 6 shows, immediately after administration of F-1,6-DP there is as a rule an increase in phosphotriose content. In the course of an hour the phosphotriose content approaches the normal level, which coincides with the disappearance of F-1,6-DP from the blood stream. The extremely small change in phosphotriose of the blood suggests that glycolysis is not the main pathway of F-1,6-DP conversion. However, the fact cannot be ignored that with the high reactivity of phosphotriose it is unlikely that there would be a significant accumulation in the blood. It was therefore deemed appropriate to determine the level of the much more stable lactic acid, the final product of glycolysis.

Changes in lactic acid content of the blood after administration of fructose-1,6-diphosphate. In the majority of experiments (five out of eight, Table 7), soon after administration of F-1,6-DP (10 minutes) there was a very slight increase in lactic acid which was followed by a decrease, and in three of five experiments the lactic acid content had fallen below the original level in 2 hours. In a smaller number of experiments (three out of eight), there was a gradual decrease after administration of F-1,6-DP. It must be pointed out, however, that in these experiments samples were not taken immediately after administration, and possibly the initial rise was missed for this reason.

In spite of the variability of results, two conclusions are possible: 1) The increase in lactic acid, which was not observed in all cases, is very slight and of short duration; 2) The insignificant increase in phosphotriose and in lactic acid indicates that the role of the glycolytic pathway in the utilization of F-1,6-DP is extremely small.

In vitro experiments on conversions of fructose esters in the blood. These experiments were designed with a two-fold purpose, first, to determine where hydrolysis of F-1,6-DP and F-6-P occurs, in the blood or in other tissues, and second, to determine the pathway of further conversion of F-6-P (Table 8). As was shown, glycolytic degradation does not play a large role in the disappearance of fructose esters, and hydrolysis of F-6-P does not proceed further to any great extent.

As Table 8 shows, after one hour's incubation of blood with F-1,6-DP there are no substantial changes in P_{inorg} or in fructose content. In experiments with F-6-P, the level of P_{inorg} remains practically constant, while the level of fructose falls markedly. The constant P_{inorg} level

in both groups of experiments indicates that in the course of an hour, at least, neither F-1,6-DP nor F-6-P is hydrolyzed Therefore, the extremely rapid in vivo hydrolysis of F-1,6-DP occurs not in the blood but in other tissues.

The fact that in the experiments with F-6-P the unchanged level of P_{inorg} is accompanied by a sharp drop in fructose content indicates that F-6-P is isomerized to glucose-6-phosphate (G-6-P). It is well known that this conversion, especially when mediated by phosphohexoisomerase, proceeds with ease. In order to estimate the rate of isomerization in our vitro experiments, it must be taken into account that an equilibrium is reached when 70% of the F-6-P is isomerized. Calculations show that in our vitro experiments (in which concentrations used were similar to those used in vivo experiments) 50-75% of the total possible amount of F-6-P is converted to G-6-P.

The main pathway of F-1,6-DP conversion is, therefore, hydrolysis to F-6-P and its isomerization of G-6-P.

Excretion of fructose esters in the urine after administration of F-1,6-DP. The question of the degree of utilization of F-1,6-DP in the organism, which is of very great interest, could be dealt with to a considerable extent on the basis of studies of excretion of fructose esters and fructose in the urine.

Results of experiments on this aspect of the problem are presented in Table 9.

Parallel chromatographic experiments showed that after administration of F-1,6-DP it appears in the urine; F-6-P and free fructose are absent. From this it could be assumed that all or nearly all the fructose was in the form of F-1,6-DP. On this assumption we calculated what part of the administered F-1,6-DP is recovered in the urine. Results of these rough calculations, which are probably somewhat high, are given in the last two columns of Table 9.

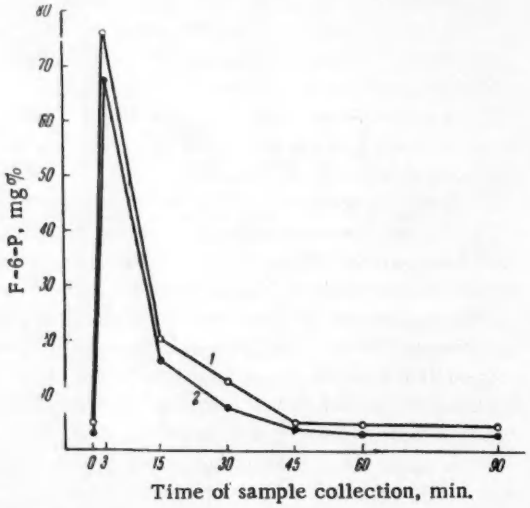


Fig. 3. Curves showing changes in F-6-P content of blood after its administration. Figures on curves indicate experiment numbers.

TABLE 6. Changes in Phosphotriose Content of the Blood (in Mg% P) after Administration of F-1,6-DP.

Time of sample withdrawal, min.	Expt. number 1	2	3	4
Before administration	0,0	0,0	1,14	1,14
After 3	3,43	1,25	1,1	1,40
" 15	4,29	1,2	—	—
" 30	2,57	1,5	1,5	1,70
" 45	0,0	1,2	—	—
" 60	—	0,0	—	1,30

As shown in Table 9, with the administration of F-1,6-DP at levels which create abnormally high concentrations in the blood, these being at least 80 times higher than ordinary concentrations (Table 3), 1/10-1/4 of the F-1,6-DP is recovered in the urine in the first 30-60 minutes. Further excretion in the urine is evidently curtailed as a result of the disappearance of the compound from the blood stream, as partly shown in Table 9. Thus, in our experiments approximately 3/4-9/10 of the F-1, 6-DP administered was incorporated.

DISCUSSION

The following pathways of conversion of F-1,6-DP introduced into the blood are theoretically possible: 1) excretion in the urine; 2) glycolytic degradation of F-1,6-DP through trioses and other products to lactic acid; 3) hydrolysis of F-1,6-DP to F-6-P followed by: a) hydrolysis to free fructose; b) isomerization of F-6-P to G-6-P which is further metabolized.

Although the experiments described do not provide a complete picture of possible conversions and their relative importance, they do afford the opportunity to judge which pathways are the major ones, which are secondary, and which are not involved at all.

As shown in Table 9, excretion of F-1,6-DP in the urine during the first 30-60 minutes accounts for about 1/10-1/4 of the amount administered. Subsequently there is no excretion, which corresponds to the disappearance at this time from the blood. In view of the fact that the amount of F-1,6-DP administered increases its concentration in the blood 80 times above the normal level, it may be considered that the organism possesses great capacity to utilize this metabolite, 3/4-9/10 being incorporated. Lucareli [4] observed no excretion after administration of a 100 mg/kg dose, and in our experiments a dose only five times as great, 0.5 g/kg, resulted in a comparatively small loss in the urine. Beloff-Chain et al. [20], who studied the metabolism of G-6-P and G-1-P administered to rabbits intravenously at a dose level of 1 g/kg, found that excretion accounts

TABLE 7. Changes in Lactic Acid Content of the Blood after Administration of F-1,6-DP (in % of Original Level)

Time of sample withdrawal, min.	Expt. number							
	1	2	3	4	5	6	7	8
Before administration	100	100	100	100	100	100	100	100
After 10	180	—	208	79	145	227	95	39
" 30	192	132	134	34	79	173	75	17
" 60	92	46	128	50	66	227	44	17
" 120	40	39	136	31	55	145	14	28

TABLE 8. Phosphatase and Phosphohexoisomerase Activity of Blood during Incubation with F-1,6-DP and F-6-P (in μg/ml)

Time of sample withdrawal min.	F-1,6-DP				F-6-P			
	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	P_{inorg}	fructose	P_{inorg}	fructose	P_{inorg}	fructose	P_{inorg}	fructose
Before expt.	293	1710	66	2010	23	1860	72	1606
After 30	293	1710	63	1950	21	1560	74	1238
" 60	293	1680	66	2010	21	1230	75	780

TABLE 9. Excretion of Fructose Phosphates in the Urine after Administration of F-1,6-DP

F-1,6-DP administered, mg	Time of urine collection	Fructose ester content μg fructose/ml	Amount of urine, ml	Total excreted fructose esters, mg	F-1,6-DP recovered	
					mg	% of administered cpd.
800	After 60 min.	3363	11	37,0	87,7	11
	" 3 hrs. 30 min.	88	7	0,6	1,4	0,2
1100	" 30 min.	3393	23**	78,0	241,8	22,0
	" 1 hr. 30 min.	1143	12	24,0		
1400	" 60 min.	3637	38	138,0	327,6	23,4

for 40 and 30% of these metabolites respectively.

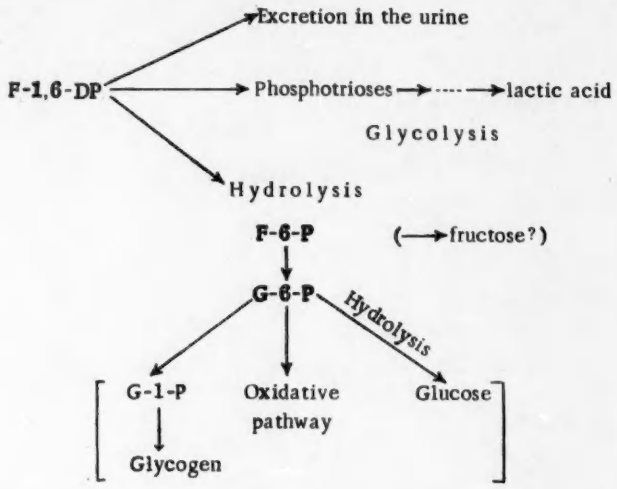
Glycolytic degradation of administered F-1,6-DP proceeds to a very small extent, as shown by the small increase in phosphotriose content (Table 6) and in lactic acid content, the latter being followed by a decrease (Table 7).

Hydrolysis of F-1,6-DP to F-6-P is evidently the first main step in the utilization of this metabolite. This is shown by two lines of investigation, the results of which are consistent with each other: the direct determination of F-1,6-DP in the blood, and the calculation based on changes in P_{inorg} content of the blood (Tables 2 and 3) which shows that in the first three minutes after administration approximately 1/4 of the F-1,6-DP is hydrolyzed. Hydrolysis does not occur in the blood but in other tissues, where it is very rapid. Similarly, Beloff-Chain et al. [20] observed that G-6-P and G-1-P are not hydrolyzed in the blood. Hydrolysis evidently stops at the F-6-P stage, since free fructose was found neither in the blood nor in the urine.

Conversion of F-6-P to G-6-P is the second main step in the utilization of F-1,6-DP, as shown by the rapid disappearance from the blood of fructose esters coincident with the maintenance of P_{inorg} levels (Table 8). The comparatively slow hydrolysis of F-6-P introduced into the blood stream, which is manifested by the slow rise in P_{inorg}, is indirect evidence of its isomerization to G-6-P. These data are in accord with those of Cori et al. [21] obtained in experiments on which liver sections were incubated with fructose phosphates. They found that fructose disappeared much more rapidly than organic phosphate because of the greater activity of phosphohexoisomerase as compared with phosphates.

Further conversions of G-6-P were not within the domain of this study, nor was the subject of the partici-

CONVERSIONS OF F-1,6-DP INTRODUCED INTO THE BLOOD STREAM



Note: Heavy type denotes the main pathway of conversion; ordinary type, the processes of less importance. Enclosure in parentheses with a question mark denotes a process we were unable to demonstrate, although it is theoretically possible. Processes within brackets are those with which we were not directly concerned, but according to published information they undoubtedly occur.

pation of various organs in the processes described. It may be assumed, however, that G-6-P can be converted to G-1-P and then to glycogen or degraded oxidatively, or finally, hydrolyzed. These possibilities are indicated schematically.

In evaluating the foregoing data, it should be emphasized, first, that even with the administration of large doses of F-1,6-DP the organism is able to utilize the major part of the metabolite and, second, that in the organism there are mechanisms which ensure, in general, an extremely economical utilization of metabolites. For example, we were unable to detect dephosphorylation of F-6-P. The same economy of utilization is manifested by the extremely small amount of glycolytic degradation. The scheme presented summarizes all the data obtained in this laboratory on the fate of F-1,6-DP introduced into the blood stream.

SUMMARY

In order to follow up by chemical analysis and by chromatography the fate of fructose-1,6-diphosphate (F-1,6-DP) and of fructose-6-phosphate (F-6-P) injected into rabbits(0.5 and 0.4 g/kg respectively), we have investigated the changes in inorganic phosphate, F-1,6-DP, reducing compounds, phosphotrioses and lactic acid in the blood, the excretion of fructose phosphates and free fructose in urine, and the transformations of fructose phosphates in the blood in vitro experiments.

It was shown that the major part of F-1,6-DP administered to the animal is used up by the organism. The main pathway is hydrolysis of F-1,6-DP to F-6-P, which takes place not in the blood but in other tissues, and its conversion to glucose-6-phosphate. Glycolytic breakdown of F-1,6-DP occurs on a small scale. No hydrolysis of F-6-P to fructose upon administration of F-1,6-DP could be detected.

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INTRACELLULAR DISTRIBUTION OF THE COMPONENTS OF THE ACETYLATION SYSTEM IN PIGEON LIVER CELLS

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The enzyme system of acetylation is a complex multicomponent system. The process of acetylation consists of at least two stages: 1) primary activation of inert acetate (or its generation from more complex precursors) with formation of acetyl – coenzyme A; 2) transfer of active acetate to an acceptor, i.e., transferase reaction. Formation of active acetate is mediated by any of a number of enzymes, depending on the original substrate, the acetyl donor. It is also obvious that there should be a multiplicity of catalysts for the transfer of activated acetate to one of the numerous possible biological acceptors.

Inasmuch as both stages are mediated by groups of enzymes, and coenzymes are also involved, it is natural to expect a complex intracellular localization of acetylation activity. The role of individual subcellular structures in acetylation is of great interest, and this is the subject of this study.

METHODS

Pigeon liver was fractionated by centrifugation at low temperature into nuclei, mitochondria, and cytoplasmic supernatant according to the method of Hogeboom et al. [1]. Each fraction was tested for its ability to acetylate sulfanilamide (method of Sytinskaya [2]) and to form acethydroxamic acid [3]. Fresh preparations were used soon after fractionation as well as enzyme extracts obtained by treatment with acetone [4]. Each fraction was assayed for coenzyme A content, expressed in Kaplan – Lipmann units [4]; the cofactor's role in the reaction system was determined by measurement of acetylation after its removal by autolysis and comparison of the results obtained with those using autolyzed preparations to which the cofactor had been returned. Coenzyme A content and acetylating activity were also calculated on the basis of 1 mg of protein (biuret method).

RESULTS

Experimental samples were prepared according to the directions of Kaplan and Lipmann [4]; the following compounds were incubated with the fraction tested (in μ moles / ml of sample): ATP, 0.363; sodium acetate, 73.2; sodium citrate, 26.2; sufanilamide, 0.393; cysteine, 10; $NaHCO_3$, 80. This combination provides the most favorable conditions for activation and transfer of acetate to sulfanilamide, insuring an adequate substrate and energy supply and also the maintenance of SH-enzymes in an active reduced state.

In Table 1 are presented data on the comparative acetylating activities of whole homogenate and the fractions obtained from it, nuclei, mitochondria, and cytoplasmic supernatant.

Results show that the whole homogenate has the greatest capacity to acetylate sulfanilamide. Acetylation values range from 4.5 to 15.5 μ grams sulfanilamide per mg protein in 30 minutes, with an average of 7.1 μ grams. The cytoplasmic supernatant also possesses a high activity, and in a number of cases its activity bears comparison with that of the whole homogenate. In view of this, it could be expected that the activity of nuclei and mitochondria would be low, and it was in fact practically absent in mitochondria In nuclei it was somewhat higher, though considerably lower than that of the whole homogenate.

The absence of activity in mitochondria and the weak activity of nuclei can be explained in various ways. It is possible that the mitochondria and nuclei lack both coenzyme A and the necessary enzyme systems. It is equally possible, however, that only one component of this complex multicomponent system is lacking. In the nuclei of rat liver cells, for example, there is no deficiency of the coenzyme itself [5].

It was possible to establish the primary cause of the inactivity of nuclei and mitochondria by supplying these fractions either with an excess of coenzyme A (preparation of 95% purity) or with an excess of the apoenzyme of acetylation in the form of an autolyzed extract of acetone powders prepared according to Kaplan and Lipmann [4]. As a rule, addition of coenzyme A had no effect on the acetylating activity of nuclei and mitochondria. Addition of an apoenzyme preparation to mitochondria, on the contrary, markedly stimulated the formation of acetyl sulfanilamide (Table 1). This convincingly shows that mitochondria contain significant amounts of coenzyme but lack all or part of the enzyme system. It is interesting that an apoenzyme preparation had no effect on the acetylating activity of nuclei. The reason for this is, however, not the absence of coenzyme

TABLE 1. Formation of Acetyl Sulfanilamide in Whole Homogenate of Pigeon Liver and in Individual Fractions

Expressed as micrograms acetyl sulfanilamide per mg protein.

Date	Homogenate			Supernatant			Mitochondria		Nuclei	
	OS	OS + coA	OS+ mitochondria	OS	OS + coA	OS+ mitochondria	OS	OS + autolyzate	OS	OS + autolyzate
May 21	8,4						1,4	8,0		
May 27	11,6						0	6,5		
June 15	4,7	9,0					0,64	7,9		
June 17	10,9	14,0					0	9,4		
June 18	11,8	14,0					1,7	9,6		
June 19	15,5	15,9					0	7,1		
June 26	4,7	6,7					0	8,6	1,0	1,40
June 29	11,4	14,3					0	8,0	2,6	2,50
July 6	10,0	10,0					0,9	8,2	1,0	1,50
July 7	11,4	12,6					0	7,8	2,0	1,70
Sept. 8	12,8	11,4		9,4	9,4		2,4	5,6	3,7	3,80
Sept. 30	5,5	7,7	5,5	3,8	7,8	8,3				
Oct. 2	6,5	8,4	4,5	5,0	9,2	6,2				
Oct. 5	4,7	7,0	3,4	5,0	7,7	6,1				
Oct. 7	5,5	8,4	6,1	5,5	7,5	7,5				
Oct. 12	4,5	7,2	5,2							
Oct. 18	4,8	9,1	6,0	6,0	10,1	7,5			0	0
Nov. 19	6,0	8,6	6,8	6,0	9,5	7,5			1,5	1,20
Nov. 20	4,9	7,4	5,6	6,8	8,5	8,7			0,9	1,00
Nov. 23	12,4	12,4	9,8	5,2	6,4	6,0			0,5	1,80
Average	7,1	10,2	5,8	5,9	8,4	7,2	0,63	7,8	1,5	1,7

Note. Composition of original sample (OS): 1 ml of preparation tested together with (in μ moles): ATP, 0.363; acetate, 73.2; citrate, 26.5; cysteine, 10; $NaHCO_3$, 80; sulfanilamide, 0.393. The homogenate and supernatant were incubated 30 minutes, the nuclei and mitochondria 2 hours, at 37°.

A, but the poor permeability of the nuclear membrane to added enzymes or to sulfanilamide. We were easily convinced of this as a result of studies with acetone powders which had been extracted with a soda solution. In this case, in which the permeability factor was eliminated, it was found that the preparations contain significant amounts of coenzyme A, and that when an apoenzyme preparation is supplied they carry on a vigorous acetylation. Extracts of acetone powders of mitochondria behave in a similar manner.

This assumption is still more strikingly confirmed by a study with autolyzed extracts of nuclei and mitochondria. Autolysis leads to the destruction of coenzyme A, and even when an apoenzyme preparation is added the ability to acetylate is absent (Table 2).

Experiments on fresh nuclei and mitochondria (Table 2) and on extracts of them indicate that the enzyme component is the limiting factor in the acetylation of sulfanilamide by the isolated fractions, while coenzyme A is present in adequate amounts. This is, however, not the complete picture. The enzyme part of the acetylation system is complex and comprises not one but several active proteins. Do the nuclei and mitochondria lack all the protein components or only some of them?

This question could be answered, at least in part, by a comparison of acetylation of sulfanilamide and hydroxylamine. As is well known, hydroxylamine can be acetylated by an incomplete enzyme system. Only the first step, activation of acetate with formation of acetyl – coenzyme A, is enzymatically mediated, transfer of the acetyl residue to hydroxylamine being non-enzymatic in contrast to the transfer to sulfanilamide.

It was shown that in the presence of all the necessary compounds (see note to Table 3), nuclei and mitochondria vigorously accumulate acethydroxamic acid. This indicates the presence in these fractions of at least part of the acetylation system: that part which mediates the activation of acetate. At the same time, the specific enzyme which catalyzes the transfer of acetate to sulfanilamide is absent. The acetylation which occurs in the absence of coenzyme A is evidently due to incomplete destruction of the coenzyme in autolysis.

The presence in the mitochondria of enzymes which mediate acetate activation is in accord with the well-known fact of the high oxidative capacity of these bodies. The presence of these enzymes in nuclei may be related to some other function.

These data indicate a complex intracellular distribution of the enzymes involved in acetylation. Coenzyme A itself is not concentrated exclusively in a single subcellular entity. It occurs in the mitochondria, the nuclei, and the cytoplasmic supernatant. The

TABLE 2. Acetylation of Sulfanilamide in Extracts of Acetone Powders Obtained from Nuclei and Mitochondria of Pigeon Liver

Expressed as μ moles acetyal sulfanilamide per sample.

Unautolyzed extracts		Autolyzed extracts		Unautolyzed extracts		Autolyzed extracts	
OS	OS + apo-enzyme prep.	OS	OS + apo-enzyme prep.	OS	OS + apo-enzyme prep.	OS	OS + apo-enzyme prep.
Mitochondria				Nuclei			
0,02	0,09	0,00	0,00	0,00	0,10	0,00	0,00
0,04	0,07	0,00	0,00	0,00	0,09	0,00	0;00
0,01	0,09	0,00		0,00	0,10		
0,01	0,06	0,00		0,01	0,09		
0,01	0,07			0,01	0,07		
0,01	0,10			0,00	0,10		
				0,01	0,11		

Note. Composition of original sample (OS): 0.3 ml extract of acetone powders; remaining components as indicated in Table 1. Samples incubated 2 hours at 37°.

TABLE 3. Acetylation of Hydroxylamine and Sulfanilamide (in μ Moles per Sample) by Autolyzed and Unautolyzed Extracts of Acetone Powders from Nuclei and Mitochondria of Pigeon Liver.

Unautolyzed extracts	Autolyzed extracts			Unautolyzed extracts	Autolyzed extracts		
	Acetylation				Acetylation		
hydroxylamine	hydroxylamine		sulfanilamide	hydroxylamine	hydroxylamine		sulfanilamide
OS	OS	OS + coA	OS + coA	OS	OS	OS + coA	OS + coA
Mitochondria				Nuclei			
0,80	0,47	1,07	0,00	0,20	0,00	0,34	—
0,60	0,34	1,14	0,00	0,10	0,06	0,87	0,00
0,48	0,00	0,54	0,00	0,27	0,20	0,77	—
0,60	0,47	1,20	0,00	0,13	0,46	0,67	0,00
0,80	0,61	1,04	0,00	0,39			
0,89							

Note. Composition of original samples (OS): 0.3 ml extract of acetone powders; sulfanilamide, 0.393 μ moles (column 4); hydroxylamine, 100 μ moles (columns 1-3); remaining components as in Table 1. Samples incubated 2 hours at 37°.

significance of this can be easily understood if it is taken into account that coenzyme A plays a universal role in both degradative and assimilative processes. On the other hand, the absence of a complete system for acetylation of sulfanilamide in mitochondria does not contradict the view of these particles as the center of the cell's oxidative activity. The absence of an enzyme for the transfer of acetate from acetyl – coenzyme A to sulfanilamide does not exclude the existence of other acetate-transporting enzymes. Indeed, the existence of such enzymes is convincingly demonstrated by the capacity of the mitochondria to catalyze a whole series of reactions of the tricarboxylic acid cycle, including the transfer of acetate from acetyl – coenzyme A to oxalacetic acid. There are evidently many acetate acceptors for which the corresponding transferases are present in adequate amounts.

Our data on the unequal distribution of individual components of the acetylation system within the cell are in accord with the results of Aisenberg and Potter, who worked with rat liver and kidney cells [6], and also with those of French workers, who failed to detect acetylation of sulfanilamide in pigeon liver mitochondria and nuceli [7]. In addition, the unequal distribution of coenzyme A in subcellular structures has been reported [8, 9].

SUMMARY

The acetylating activity of whole homogenate from pigeon liver as well as nuclei, mitochondria and cytoplasmic supernatant was studied.

Acetylation of sulfanilamide is maximal in whole homogenate, greatly decreased in the nuceli, and practically absent in mitochondria. The activity of the supernatant obtained after separation of nuclei and mitochondria is close to that of the whole homogenate.

Extracts of acetone powders of nuclei and mitochondria form acethydroxamic acid in the presence of hydroxylamine, and when supplied with the apoenzyme of the acetylation system obtained from acetylate sulfanilamide as well.

The nuclei and mitochondria of pigeon liver contain coenzyme A and the enzymes for primary activation of acetate but lack the enzymes which catalyze acetate transfer to sulfanilamide.

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A STUDY OF SEVERAL WAYS OF INHIBITING THE PROCESS OF DEVELOPMENT OF BACTERIAL RESISTANCE

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The feasibility of the use of antibiotics and chemotherapeutic substances for the treatment of infectious diseases is accompanied by the difficulty of the appearance of bacterial resistance during the course of treatment [1-9]. One of the most essential questions of chemotherapy is to find effective antibiotics and chemotherapeutic substances and methods for their rational use, in order to prevent or inhibit the development of microbial resistance.

The concept of bacterial synergism of chemotherapeutic substances led us to undertake, in 1946, the problem of the suppression of the development of bacterial resistance by the use of chemical combinations of two or several antibacterial substances, having different chemical functions and different pharmacodynamic and antimicrobial effects. Thus, we attempted to establish simultaneous action on many links of the metabolic reactions of pathogenic organisms. On the basis of this principal, we synthesized sulfosalicylate-allylthiocyanate-streptomycin [10, 11].

Resistance of the tuberculosis bacillus Mycobacterium tuberculosis H. Ratti to the sulfosalicylate-allylthiocyanate-streptomycin is more slowly developed in vitro and in vivo than to streptomycin [12, 13]. Experiments artificially increasing the resistance of the tuberculosis bacillus to antibiotics by means of serial passage on media with increasing concentrations of antibiotics have shown that resistance to the antibiotics at a concentration of 25,000 μg/ml arises in streptomycin-containing media (beginning with a 1:6,000,000 dilution) after 19 passages and on sulfosalicylate-allylthiocyanate-streptomycin-containing media (beginning with a dilution of 1:13,000,000) much later – after 34 passages. This fact indicates that adaptation of bacterial metabolism to this antibiotic occurs more slowly and with difficulty.

The acquired resistance to sulfosalicylate-allylthiocyanate-streptomycin is shown to be unstable – it decreases after 7 passages on media without sulfosalicylate-allylthiocyanate-streptomycin to 4200 μg/ml, in contrast to acquired resistance to streptomycin, which is unchanged after 7 passages in media without streptomycin.

Clinical and experimental investigations have shown the validity of combining two or several antibacterial substances, with different chemical functions and different pharmacodynamic and antimicrobial action in order to suppress the development of microbial resistance [14, 15]. Investigations of Kalninish and Shmidt [16] also lead to the conclusion that combination of antibiotics with chemotherapeutic substances is one of the methods of inhibiting the development of bacterial resistance. Vaisberg [17] insists that selection of substances acting in combination focuses attention on their differing activities in the metabolism of pathological organisms. Following in this direction were the investigations of Gobbi and co-workers [18], using streptomycin simultaneously with p-aminosalicylic acid, investigations of Viallier and Cayré [19], associating streptomycin with isonicotinic acid hydrazide, and investigations of Kourilsky [20] using p-aminosalicylate isonicotinic acid hydrazide. It has been shown [21] that chemical combination of aureomycin and bacitracin (aureotracin) give a better bacteriostatic effect, than these preparations used separately.

It may be noted that respiration of mycobacteria in the presence of sulfosalicylate-allylthiocyanate-streptomycin differs from respiration in the presence of streptomycin (Table 1).

In sensitive strains, sulfosalicylate-allylthiocyanate-streptomycin in a concentration of 10,000 μg/ml, suppresses respiration 21%; in order to decrease respiration 28%, a 10-fold increase in the concentration of streptomycin (100,000 μg/ml) is needed [22].

In resistant strains under the same conditions sulfosalicylate-allylthiocyanate-streptomycin suppresses the respiration of bacteria, while streptomycin, on the contrary, stimulates it.

The data given in Table 1, in which the tuberculocidal action of sulfosalicylate-allylthiocyanate-streptomycin is seen to surpass by 300 times the activity of streptomycin, when compared with data on the bactericidal action of antibiotics and adaptation of bacteria to antibiotics, lead to the concept of a correlation between the degree of bactericidal action of an antibiotic and its activity on respiration of the microbe, and

TABLE 1. Change in Respiration (% of Control) of Sensitive and Resistant Strains of Mycobacterium tuberculosis H. Ratti under the Influence of Streptomycin and Sulfosalicylate-Allylthiocyanate-Streptomycin after a 2-Hour Contact.

Concentration of the preparations, μg/ml	Sensitive strain		Resistant strain	
	streptomycin	sulfosalicylate-allylthiocyanate-streptomycin	streptomycin	sulfosalicylate-allylthiocyanate-streptomycin
10	+ 3	+12	+18,9	+ 7
40	− 0,4	+23	− 8	− 6
100	− 8,7	− 5	+ 6,2	+14,4
1000	− 9,2	−10	+ 4	−20,3
10000	− 8,0	−21	− 3	−37,2
100000	−28	−	−12	−

probably a correlation between the influence on microbial respiration and the development of microbial resistance.

Continuing our investigation, we began with the fact that the development of microbial resistance could be prevented or inhibited by increasing the bacteriostatic concentration in the blood of injected chemotherapeutic substances (which is possible only with substances of very low toxicity). With this in mind, we introduced the hypothesis that for realization of highly active chemotherapeutic substances well-tolerated by the organism, it was necessary to have chemical combinations of active principles with the basic metabolites of the organism which play an important role in carbohydrate, fat, and protein metabolism. On the basis of this hypothesis, we synthesized hydrazones of isonicotinic acid hydrazide by condensation with the ketonic acids (pyruvic, oxaloacetic and α-ketoglutaric).

The hydrazones obtained (isonicotinoyl hydrazones of pyruvic, oxaloacetic and α-ketoglutaric acids) possess tuberculostatic activity approaching that of isonicotinic acid hydrazide, significantly less toxicity (4.5-10 times) and higher therapeutic index (3-6 times) [23].

Changes in the group $-NH-NH_2$ result in the formation of chemotherapeutic substances with better tolerance than isonicotinic acid hydrazide [24-27] which is the basis of the preparation of a large number of derivatives of isonicotinic acid hydrazide (phthivazide, flavoteben, isoteben) widely used in medical practice.

Experiments in vivo have shown that per os or intramuscular injection of isonicotinic acid hydrazone of pyruvic acid into guinea pigs results in higher bacteriostatic activity of the blood than injection of other hydrazones being used on a large scale in clinical practice.

In spite of the superiority of the hydrazones obtained, resistance of Mycobacterium tuberculosis H. Ratti to the isonicotinoyl hydrazone of pyruvic acid developed as quickly as in the case of isonicotinic acid hydrazide.

Experiments artificially increasing the resistance of the tuberculosis bacillus show that resistance to the drug in concentrations of 10 μg/ml arises after 8 passages in media containing isonicotinic acid hydrazide, and in media containing the isonicotinoyl hydrazone of pyruvic acid – after 10 passages. From this it follows that obtaining a high bacteriostatic threshold in blood has great significance, but it is insufficient for prevention of the development of microbial resistance.

Respiration of sensitive and resistant strains of the tuberculosis bacillus (Table 2) is inhibited insignificantly by these chemotherapeutic substances [22].

The absence of a correlation between the development of resistance and bacteriostatic threshold in blood led us to a study of the correlation between chemical structure and the development of bacterial resistance in which the basic criterion was the intensity of suppression of pathogenic microbe respiration in the presence of some active substances with low toxicity.

We prepared numerous substances for this purpose, among which are cited the following: succinyl-n-aminobenzallyl thiosemicarbazone; o-thioureidobenzalthiosemicarbazone; o-,m-,and p-dihydroxypropoxybenzalthiosemicarbazone; o-, m-, and p-allylthioureidobenzoylhydrazide; o-dihydroxypropoxybenzoyl hydrazide; mercaptopyruvic isonicotinoyl hydrazone; bromopyruvic isonicotinoyl hydrazone; levulinic isonicotinoyl hydrazone; 4-dihydroxypropoxy-3-methoxybenzal isonicotinoyl hydrazone, etc.

These substances did not suppress bacterial respiration markedly, nor did they inhibit the development of bacterial resistance.

Bodrana's investigation [28] indicated that a considerable quantity of cellulose enters into the chemical composition of the tuberculosis organism (3.6-5.5%) which brought us to a search of media of xylophagic fungi (wood-decomposing fungi) for a natural antibiotic with tuberculostatic, or chiefly tuberculocidal action. From the fungus Coniphora cerebella, from the subclass Basideomycetes, family Thelephoraceae, "conioforin" was obtained – an antibiotic with insignificant tuberculostatic, but with definite tuberculocidal, action in which the threshold of bactericidal

TABLE 2. Change in Respiration (% of Control) of a Strain of Mycobacterium tuberculosis H. Ratti Sensitive, and a Strain Resistant to Isonicotinic Acid Hydrazide (10 μg/ml) in the Presence of Isonicotinic Acid Hydrazide and Isonicotinoylhydrazone after 2-Hour Contact.

Concentration of preparations, μg/ml	Sensitive strain		Resistant strain	
	Isonicotinic acid of hydrazide	Isonicotinoylhydrazone of pyruvic acid	Isonicotinic acid of hydrazide	Isonicotinoylhydrazone of pyruvic acid
100	-13.5	-12	-9	-16
400	-26.2	-19.5	-18	-22
1000	-18.9	-16.5	-20	-25

TABLE 3. Change in Respiration (% of Control) of the Tuberculosis Bacillus in the Presence of Conioforin and Streptomycin after 2-Hour Contact.

Conioforin		Streptomycin	
μg/ml	respiration	μg/ml	respiration
5	+100	5	+10
10	+170	10	+ 4
20	+ 70	20	+ 1,2
40	— 20	40	+ 0,4
100	— 50	100	— 8,7
200	— 70	1000	— 9,2
400	—100	10 000	— 8,0
1000	—100	100 000	—28

activity was close to the threshold of bacteriostatic activity (tuberculocidal action 1:7600; tuberculostatic 1: 30,000, that is, the ratio between the threshold of tuberculocidal and tuberculostatic action was 1: 2.6 to 1: 4).

In the presence of an insiginificant number of tuberculostatic units (400 μg/ml) of conioforin, the respiration of the tuberculosis bacillus was completely inhibited and bacterial resistance did not develop (Table 3).

An experiment artificially increasing resistance of the tuberculosis bacillus to conioforin in comparison with streptomycin showed that after 10 passages on streptomycin-containing media, resistance to the latter begins at a concentration of 1200 μg/ml, while at the same time resistance does not develop in conioforin-containing media. These data indicate the importance of an approach to the threshold of bacteriostatic and bactericidal action (which leads to full inhibition of respiration of mycobacteria and prevention of the development of resistance by insignificant numbers of bacteriostatic units [29]). In other words, for the objective of suppression of the development of microbial resistance, it is necessary to obtain chemotherapeutic substances with perhaps only slight bacteriostatic activity, but with bactericidal activity closely approaching the bacteriostatic. This fact was verified by us on new substances, which occupy a narrow zone between the thresholds of bacteriostatic and bactericidal action [30-32].

Positive results were obtained in this investigation, we began with the assumption that active principles could be obtained from coniferous plants (keeping in mind that numerous tuberculosis sanatoria are located in the midst of pine forests).

The tuberculocidal action of resins and acids of the abietic acid type and also their isopropyl amines, which were obtained from pines (Picea polita, Picea excelsa, Pseudotsuga taxifolia, Pinus montana, and others) approached closely to tuberculostatic activity (1:3), as in the case of coniofrin. Artificially increasing the resistance of Mycobacterium tuberculosis H. Ratti and Mycobacterium tuberculosis $H_{37}Rv$ by increasing the concentration of pine resins, acids of the abietic type and the isopropyl amine of abietic acids was unsuccessful. These data show that sensitivity of Myco-

Properties	Mycobacterium tuberculosis H. Ratti			M. tuberculosis $H_{37}Rv$		
	resin	acid of abietic type	isopropyl amine	resin	acid of abietic type	isopropyl amine
Beginning sensitivity	1 : 320 000	1 : 320 000	1 : 320 000	1 : 560 000	1 : 320 000	1 : 560 000
Number of passages	10	10	10	8	8	8
Sensitivity after passages	1 : 180 000	1 : 180 000	1 : 180 000	1 : 560 000	1 : 320 000	1 : 560 000

TABLE 4. Change in Respiration of the Tuberculosis Bacillus under the Influence of Resins and Acids of the Abietic Type and Isopropyl Amines of the Same Acids after 2-Hour Contact.

Test preparation	Concentration, γ/ml	Respiration, % of control
Resin	10	— 7,7
	50	—42,0
	100	—76,6
	400	—76,0
Acid of abietic type	10	+ 6
	50	— 60
	100	—100
	400	—100
Isopropyl amine	10	— 21
	50	— 51
	100	—100
	400	—100

bacterium tuberculosis $H_{37}Rv$ is completely unchanged after 8 passages, and sensitivity of Mycobacterium tuberculosis H. Ratti is decreased after 10 passages from 1:320,000 to 1:180,000; that is, a negligibly small decrease.

The respiration of mycobacteria is suppressed completely by comparatively small concentrations of preparation (insignificant number of tuberculostatic units, Table 4).

SUMMARY

A correlation exists between the value of the threshold of tuberculocidal and tuberculostatic action of antibiotics and chemotherapeutic substances, on the one hand, and inhibition of respiration of mycobacteria, on the other hand; a correlation exists also between the inhibition of respiration and the development of microbial resistance to these substances.

Chemotherapeutic substances with high bacteriostatic action are less effective in suppressing the development of microbial resistance than substances with low toxicity and bactericidal activity, approaching the bacteriostatic.

Small doses of chemotherapeutic substances, having tuberculocidal action approaching the bacteriostatic, completely inhibit the respiration of bacteria, their adaptation to these substances and development of microbial resistance. In this connection, there is the prospect of suppressing the development of microbial resistance by introducing into the chemotherapeutic molecule with tuberculostatic action, certain other substances with chemical groups which markedly suppress respiration of pathogenic organisms.

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A STUDY OF THE BIOSYNTHESIS OF PROTEINS BY THE UPTAKE OF LABELED METHIONINE

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Many investigators judge the process of biosynthesis of proteins in biological systems by the uptake of labled amino acids, assuming that the latter are combined as peptide bonds.

However, it has recently been shown that amino acids are combined directly with proteins not only by peptide, but also by nonpeptide bonds. Thus, methionine and cysteine are partly combined with proteins by disulfide and adsorption bonds [1, 2]; 60-95% of the cysteine combined with protein is found in the disulfide bond portion [3, 4]. Glycine, also widely used in such investigations, is also adsorbed to a considerable degree on proteins [5,8]. In addition, labeled glycine, which exchanges with the glycine of glutathione, is mostly bound to liver protein by disulfide bonds which in turn are formed between the protein and glutathione [6, 9]. Finally, 30-80% of the phenylalanine bound by histone and insulin is adsorbed on the protein [10].

In spite of these facts, most investigators who study the uptake of amino acids in proteins use methods for treating the proteins which cannot with sufficient completeness remove amino acids bound to proteins directly by nonpeptide bonds, and therefore they cannot judge the true renewal of the protein molecule and the biosynthesis of proteins. Thus, the basic data on the uptake of labeled amino acids in isolated proteins [4, 7, 8, 11-17] were obtained using just such imperfect methods of treating proteins.

Processes are also known for splitting nonpeptide bonds between proteins and amino acids. Thus, disulfide bonds are unstable in an alkaline medium toward various reducing agents, of which the most effective is thioglycolate [18, 19], and are unstable in acid medium toward H_2O_2. [6]. Moreover, splitting of adsorption bonds is possible by treatment with ninhydrin for amino acids labeled in the carbon of the carboxyl group [6, 7], by treatment with dinitrophenol [10], and by reprecipitation of the protein from an alkaline solution [7, 8, 13]. Finally, removal of free amino acids can occur with prolonged dialysis of an alkaline solution of the protein in running water.

However, the use of each of these methods separately does not give the desired results. Thus, dialysis of a neutral protein solution [7, 10] or reprecipitation of the protein from alkaline solution, but without dialysis [7, 8], is not effective enough for breaking the adsorption bonds and removing the free amino acids. Moreover, there is evidence that hydrolysis for many hours of a labeled protein in 6 N NaOH without dialysis not only does not decrease the specific activity of the protein, but in some experiments even increases it 2-2.5 times [15]. The use of only one treatment of the protein with reducing agents in work with methionine or cysteine [1, 3] is also insufficient for removing the adsorbed and free amino acids.

The present work was carried out to choose the best method for treating proteins in order to permit estimation of the binding of labeled amino acids to proteins directly by peptide bonds and hence to permit an accurate estimation of the intensity of biosynthesis of proteins in vitro in biological systems.

METHODS

The method was worked out for application to rabbit liver homogenates and it was tested on homogenates of chick embryo tissues and on homogenates of rabbit erythrocytes. The labeled amino acid chosen was methionine-S^{35}, which permitted an estimate of the effectiveness of the treatment both for adsorbed and for disulfide bonds. The use of a sulfur-containing amino acid also permitted the employment of a relatively sensitive method for determining the relative activity of the protein by the specific activity of the sulfur isolated from it [20].

The protein was treated in the following steps: defatting, splitting of the disulfide bonds, splitting of the adsorption bonds between protein and amino acids, and complete removal of the free amino acids.

Rabbit liver was homogenized with cooling with Hanks solution [21], without phenol red and $CaCl_2$, in the ratio 1:1 (by weight). The composition of the sample was as follows: 2 ml of homogenate, 12 ml of modified Hanks solution, pH 7.4, 1 ml of solution of methionine-S^{35} which contained 0.5 mg of amino acid with total activity of 2,000,000 counts/min. The sample was incubated at 37° for three hours (in some experiments at 100°). After incubation the sample was treated with 15 ml of 10% TCA, the precipitate was separated by centrifuging and was twice washed with 20 ml of 5% TCA

with repeated centrifuging. Then the precipitate was defatted with 20 ml of acetone, alcohol, and ether, added successively, and was separated by centrifuging. A control sample, immediately after preparation, was treated with TCA and given the same treatment.

Then the disulfide bond was split by oxidizing it with H_2O_2 or by reduction with thioglycolate, cysteine, and cyanide. For oxidation the protein precipitate was treated with 5 ml of 85% formic acid and 1 ml of 26% H_2O_2. After 30 min standing at room temperature with stirring, the protein was precipitated with 6 ml of 10% TCA. For reduction, the protein was treated with 20 ml of a solution of one of the compounds in 0.05 NaOH (sodium thioglycolate 200 mg: cysteine 500 mg: KCN 200 mg), and was at once made alkaline with 3 N NaOH to an orange color with Tropeolin 0 and the sample was kept for three hours at 30° with periodic stirring. In the first half of the incubation, the protein usually dissolved, which was necessary for complete reduction of the disulfide bond. After three hours the protein was precipitated with 2 ml of 80% TCA and separated by centrifuging.

After splitting of the disulfide bond by oxidation or reduction the protein precipitate was dissolved in 20 ml of 0.05 N NaOH which contained 5 mg of unlabeled methionine and was dialyzed in a collodion bag in running tap water for 12 hours. Then 2 ml of 80% TCA was added and the precipitate was separated by centrifuging. The resulting moist protein precipitate was burned (two parallel samples) and the specific activity of the protein sulfur was determined. In distinction from the original method [20] the precipitate of benzidine sulfate was washed not by centrifuging, but by washing three times on a Schott filter No. 4 with 1 ml of ethanol.

In studying the process of binding of methionine-S^{35} by liver protein at 100° the latter did not dissolve in 0.05 N NaOH or even in 1 N NaOH, due to denaturation. Therefore, in these experiments, for more complete splitting of the disulfide bond, its oxidation was followed by reduction with thioglycolate in an alkaline medium. This was possible because the denatured protein after treatment with formic acid and H_2O_2 again became soluble in 0.05 N NaOH.

In studying the uptake of methionine-S^{35} in proteins of rabbit erythrocytes treatment of the protein differed in that the protein was dissolved in 0.1 N NaOH and did not have to be defatted.

RESULTS

Considering that part of the amino acids are bound with the protein by nonpeptide bonds, it was interesting to determine just how much methionine-S^{35} which was bound to liver protein was combined by peptide linkages. For this purpose, part of the experimental samples were treated by the simplest and most widely used method (washing with 5% TCA with unlabeled methionine 10 times, treating with alcohol and ether), another part was treated as described above by the complex method, but without the step of splitting the disulfide bond, and finally, the remainder of the samples by the full complex method. This treatment permitted a quantitative differentiation of the forms of bonds between the proteins and the amino acids. The results of these experiments with rabbit liver homogenate and chick embryo tissues, and also with the hemolyzate from rabbit erythrocytes are given in Tables 1 and 2.

The data show that of the total amount of methionine bound by liver protein, only 12% of the amino acid is combined by peptide bonds (thioglycolate treatment), 34% by disulfides, and 54% by adsorption. In the embryo protein, 20% of the amino acid is held by peptide bonds, 53% by disulfides, and 27% by adsorption. Of all the methionine-S^{35} combined with the hemolyzate protein, the portion of peptide bonds is 14%. Thus, only 12-20% of the amino acid bound to the proteins is held by peptide bonds, and hence only this portion can characterize the process of protein biosynthesis.

Estimation of the various processes for splitting the disulfide bonds (Table 1) indicates that thioglycolate treatment is most effective. However, considering the relative scarcity of thioglycolate, we must mention that treatment with cysteine or formic acid and H_2O_2 also gives satisfactory results. The observed effectiveness of thioglycolate, cysteine, and cyanide for the disulfide bond agrees with the data of Goddard and Michaelis [18] and of Mirsky and Anson [19].

Goddard and Michaelis suggested that in splitting disulfide bonds by reducing agents at 30° there may also be some splitting of the peptide bonds due to the action of the alkaline medium (in our experiments the pH was about 12.5). However, if the protein solution is only 0.05 N NaOH without addition of the reducing agent and is incubated at 30° as described above, then according to our data such treatment is practically ineffective not only for peptide bonds, but also for disulfide bonds (Table 1).

Hence, there is no basis for fearing that the use of our complex treatment of proteins will give low results because of partial splitting of peptide bonds.

In comparing the activity of control and experimental samples it becomes evident that the peptide bond between methionine-S^{35} and proteins is formed only in the incubation. In the control samples formation of peptide bonds is scarcely found and the residual activity of these samples can be considered zero. As to the nonpeptide bonds, they are formed both during treatment (control samples) and at the time of incubation (experimental samples). However, during incubation there is more intensive formation of these bonds than there is during treatment. For the adsorption bonds this can be explained by the increased amount of amino acids adsorbed by the protein because of the great

TABLE 1. Characteristics of the Bonds of Methionine-S^{35} with Proteins of Homogenates from Rabbit Liver and Chick Embryo Tissues

Method of protein treatment	Unsplit bonds between amino acid and protein	Specific activity of protein S, counts/min/μg S			
		rabbit liver		chick embryo	
		control	expt.	control	expt.
Splitting by TCA, defatting	Adsorption, disulfide, peptide	1,17	7,17	2,10	9,42
Defatting, splitting adsorption bonds, removal of free amino acids	Disulfide, peptide	1,22	3,32	2,13	6,91
Complex treatment in splitting disulfide bonds, hydrolysis (in 0.05 N NaOH)		1,01	3,29	2,19	7,13
reduction by thioglycolate, cysteine, cyanide, oxidation (hydrogen peroxide with formic acid)	Peptide	0,11	0,90	0,09	1,86
		0,12	1,33	0,42	4,51
		0,90	2,41	1,11	6,61
		0,30	1,21	0,44	4,06

TABLE 2. Combining of Methionine-S^{35} with Protein of Rabbit Erythrocyte Hemolyzate (in counts/min/μg S of the protein) Composition of sample: 2 ml of hemolyzate (1:1), 12 ml of Hanks solution, 1 ml of solution which contains 0.25 mg of methionine-S^{35} with total activity 600,000 counts/min. Incubation 3 hours at 37°

Sample	Wash protein with TCA, defat	Complex treatment of protein with thioglycolate
Control	2.13	0.26
Experiment	7.16	0.99

TABLE 3. Binding of Methionine-S^{35} to Protein of Rabbit Liver Homogenate at 37° and 100° (counts/min/μg S of the protein)

Sample	Treatment of Konikova et al. [12]	Complex treatment used for splitting disulfide bons		
		Formic acid and H_2O_2	Thioglycolate	Formic acid with H_2O_2 and thioglycolate
Control	1.09	0.47	0.10	0.10
Experiment at 37°, 3 hours	8.32	1.44	0.56	0.55
Experiment at 100°, 3 hours	16.36	4.01	2.15	1.86

length of the reaction time of the protein with the amino acid, and for the disulfide bonds by the transformation of methionine into cysteine [3] or homocysteine which easily combines with the protein by disulfide bonds.

Konikova, Kritsman, and Samarina [12] showed that at 100° the total amino acid is bound by protein of the liver more intensively than at 37°. However, the treatment of the protein which they used could not exclude an increased binding of amino acid by the denatured protein because of adsorption and disulfide bonds [22]. In this connection we have carried out analogous experiments with treatment of the protein by the complex process in different variants, and also for comparison, by the process used by these authors (10 times washing with 5% TCA with unlabeled methionine, treatment with alcohol and ether) [12].

Table 3 shows that in treatment by the Kritsman process the increase in activity of the protein incubated at 100° over the activity of the protein incubated at 37° is chiefly due to increased binding of methionine by the denatured protein by adsorption and disulfide bonds. This agrees well with the ideas given above. However, it is evident from these experiments that at 100° methionine-S^{35} is also combined with liver proteins by peptide bonds in greater degree than at 37°. Thus the mere fact of strengthened "true" binding of amino acids by liver protein at 100° is confirmed. Nevertheless it should be mentioned that the increased binding at 100° occurs chiefly because of amino acids bound to protein by nonpeptide bonds, and to a lesser degree because of amino acids bound to protein by peptide bonds.

DISCUSSION

The proposed complex method of treating proteins after their reaction with methionine-S^{35} assures removal of all amino acids bound by adsorption and disulfide bonds and hence permits estimation of the binding of methionine by the peptide bond alone to proteins; obviously this can be applied also for other labeled amino acids.

Of the total amount of methionine-S^{35} bound by proteins of rabbit liver and erythrocytes and by chick embryo tissues, only 12-20% of the amino acids are bound by the peptide link and the other 80-88% by adsorption and disulfide bonds. In various reactions with completely or partly denatured proteins, the portion of amino acid bound by peptide bonds rises sharply. All this shows that the widely used method of washing out the protein is not suitable for studying the true renewal of the protein molecule, the biosynthesis of proteins. The suggested method is free from these objections.

Comparison of the biosynthesis of proteins in embryonic and some mature tissues, according to the data of binding methionine-S^{35} by peptide bonds, shows that this process in embryonic tissue is considerably more intensive (Tables 1 and 2). If we calculate the sulfur content found in the proteins of the objects studied (liver protein 0.56, hemolyzate 0.34, embryo 0.77%), the difference becomes still greater. This agrees with the known data on the higher activity of protein biosynthesis in embryonic tissue than in mature tissue [23]. Comparison of the corresponding results obtained by using the ordinary treatment of the protein does not indicate a clear difference between them in such experiments. This is still further confirmation of the expediency of using our method for study of the biosynthesis of proteins.

In the work of Konikova et al. [12] and also in our work there is shown the presence of intensive uptake of amino acids into protein homogenates of rabbit liver at 100°. The question arises whether to consider the process enzymatic, as Samarina and co-workers attempted to do [13]. Obviously such an assumption is unlikely. The citations of Samarina and co-workers on the retention of activity of some proteolytic enzymes at 100° [24] are groundless. In the work of Emmart and Longley [24] and other work summarized by Northrop, Kunitz, and Herriott [25] it was actually shown that some proteolytic enzymes and hyaluronidase are stable to short treatment (less than 2-3 hours) at high temperature. However, at the moment of action of the high temperature, for example, boiling, the enzymes are completely inactivated and their reactivation is possible only by later cooling of their solutions. Hence, under the conditions of our experiments, and also the experiments of Samarina and co-workers, during the 2-3-hour incubation at 100° the proteolytic enzymes should be inactivated. The suggestion of Samarina and co-workers of the existence of a specific enzyme system which catalyzes the process of uptake of amino acids into proteins and which retains its activity at 100° is also unlikely. It is interesting to note that Brunish and Luck [7] and Cornwell and Luck [10] obtained analogous results, in distinction from Samarina and co-workers, finding no binding with retention of enzyme activity at higher temperatures, especially in experiments with isolated proteins.

The more intense uptake of amino acids into proteins at 100° than at 37° shown by the use of imperfect methods [7, 12] can be explained on the basis of an increased portion of amino acids bound by nonpeptide bonds to the denatured protein [22]. This is also partly confirmed by the data of Cornwell and Luck [10] who showed that phenylalanine is bound with histones at 122° in considerably greater quantity than at 37°. Thereupon there is an increase in the portion of adsorbed amino acids bound by stronger bonds, probably peptide.

In the present work we have established a more intense binding of methionine-S^{35} to liver proteins by peptide bonds at 100° than at 37°. This can evidently be explained as follows: The reaction of formation of peptide bonds is endothermic ($\Delta H > 0$) and reversible [26].

After three hours incubation at 37° there is established in the system a dynamic equilibrium [1, 14]:

[protein] + [amino acid] ⇌[protein-amino acid].

When the temperature is raised from 37° to 100°, in accord with the Le Chatelier-van't Hoff principle there will be a shift in the equilibrium of this endothermal reaction to the right. The attainment of a new equilibrium concentration evidently depends in a nonenzymatic way on the hastening of both the forward and reverse reactions. In the final account this leads to an increased amount of amino acids combined with protein.

Also, in the denaturation of the protein there occurs partial splitting of hydrogen bonds between amino and carboxyl groups on the surface of the protein molecule [27] and unfolding of the protein molecule. Hence, under the conditions of our experiment, there can be liberated in the denatured protein the earlier-bound amino and carboxyl groups which can form peptide bonds with amino acids. This can increase the concentrations of free amino and carboxyl groups of the protein which share in the reaction, and hence cause a further shift in the equilibrium of this reaction to the right. This agrees with the suggestion of Brunish and Luck [7] of the change in configuration of the protein at 100° with "uncovering of reactive centers."

It is also important to observe that the findings of a number of investigators [7, 8, 11, 14, 17] of the total binding of labeled amino acids by isolated proteins at 37° does not show that amino acids in these experiments are bound by peptide links, and some of these authors even deny this categorically. Moreover, when Samarina and co-workers [13] attempted to show the presence of peptide bonds by using a differential treatment of the protein, they observed that amino acids combined at 37° with the proteins of serum, plasma, and with deoxypentosenucleohistones only by nonpeptide bonds. The relation observed by Kritsman and co-workers [14] between the quantity of amino acids bound by myosin and serum protein and the concentration of free amino acids was not linear, as the authors asserted, but could be expressed by the adsorption isotherm of Freundlich (calculating it by their own numerical data). This strongly indicates that in studies of this type it is essential to use such methods for treating proteins as will avoid adsorption. It is interesting to observe that Orekhovich et al. [28] did not find binding of labeled amino acids by isolated proteins of plasma at 37° under conditions in which Kritsman et al. [11] found this process. Cornwell and Luck [10], working especially on peptide bonds, showed that phenylalanine practically does not combine at all with histones at 37° over prolonged incubation periods (from 2 to 24 hours). Levyant, Levchuk, and Orekhovich [29] in vivo experiments on rats also failed to find autocatalytic uptake of labeled methionine into blood albumin, contrary to Konikova and Kritsman [30]. The authors showed that uptake of methionine into albumin occurred in the liver during biosynthesis of the protein. Finally, Konikova and co-workers [15, 16] in a study of the type of bonds formed in the uptake of amino acids by proteins, concluded that these bonds, like the protein itself, were split in incomplete hydrolysis under the action of acid or alkali or by trypsin. On this basis they concluded that the bonds between protein and labeled amino acid had a peptide character. However, such a conclusion does not have a sound basis, since, first, in protein hydrolysis it is not only peptide bonds which are split, but also adsorption-disulfide bonds, because of the destruction of the protein molecule as a whole. Second, in the experiments carried out with decreasing total activity of the labeled protein during its hydrolysis, the specific activity either was practically unchanged, or increased two to three times. This is evidently the result of reverse adsorption of the labeled amino acid freed in the hydrolysis and demands, in all cases of this sort of study, a dialysis of the hydrolyzate before precipitating the remaining protein with TCA. It is interesting to see that, although they were aware of the impossibility of judging the nature of bonds by their relation to nonenzymatic hydrolysis, Konikova, Kritsman and co-workers for some reason considered it possible to estimate this on the basis of experiments with enzymatic hydrolysis [16].

Hence, on the basis of present work it is still impossible to consider as experimentally established the ability of isolated proteins to combine by peptide bonds with amino acids at 37°. It is possible that this will be confirmed in the future. However, there is no basis at all for an attempt to explain this process by autocatalytic activity of the protein [30]. The combination of isolated proteins and labeled amino acids by peptide bonds can be considered as the result of a nonenzymatic chemical reaction:

[protein] + [amino acid] ⇌[protein-amino acid]

in which a slight concentration of the reaction product can be detected because of the high sensitivity of the method of labeled atoms. Therefore we cannot agree with the assertion of Konikova and Kritsman that they have already shown "the ability of isolated proteins to utilize amino acids for their construction", and that they have established the presence of a process of autocatalytic self-metabolism of proteins [30].

SUMMARY

The proposed method for treatment of proteins permits estimation of the binding of methionine-S^{35} by peptide bonds to proteins and hence offers the possibility of studying the process of biosynthesis of proteins in some biological systems under various conditions, especially under the action of denaturing agents.

The widely used method of treating proteins by washing with TCA, alcohol, and ether is not suitable for studying the biosynthesis of proteins, since in this

method only 12-20% of the amino acid bound by the protein occurs as peptide bonds.

At 100° methionine-S^{35} combines with rabbit liver proteins more intensively than at 37°, chiefly because of the increased amount of amino acid bound by non-peptide bonds and to a lesser extent because of amino acids bound to the protein by peptide bonds.

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METABOLISM OF POLYPHOSPHATES AND OF SOME OTHER PHOSPHORUS COMPOUNDS IN THE DEVELOPMENT OF FRUITING BODIES OF THE MUSHROOM Agaricus bisporus L.

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In our previous work [1] it was shown that in fruiting bodies of the double-spore mushroom (Agaricus bisporus L.) quite a considerable number of polyphosphates are present. In a portion of these compounds, up to 50% of the total phosphorus of the fruiting bodies is located. The main bulk of the polyphosphates (80-90% of their total quantity) are found in the acid-soluble fractions and include the whole gamut of polyphosphates from pyrophosphate to comparatively highly polymerized condensed phosphates.

However, our earlier data did not establish whether the polyphosphates are synthesized in the fruiting bodies of the mushrooms themselves or whether they are formed in the mycelium and transported as polyphosphates to the fruiting body. In this connection, the question of the physiological role of polyphosphates in these fruiting bodies arose. Are they a form of transport of phosphorus from the mycelium through the fruiting body into the spores, or do they play some sort of role in the life activity of the fruiting body itself?

In the present work we attempted (if only in some degree) to approach a solution to these questions both by the usual analytical methods and by the use of radioactive P^{32}.

MATERIALS AND METHODS

The subjects of investigation were the fruiting bodies and spores of the double-spored mushroom Agaricus bisporus L.) strain F_1. We obtained the fruiting bodies of various ages from the greenhouse hotbeds of the "Marfino" farm. Chemical analysis was undertaken in the following: the incompletely differentiated fruiting body 1.5 days old, which was a small protuberance on the mycelium; 2-day-old fruiting bodies, differentiated as to cap and stalk, but in which the process of formation of the gills still was not completed; fully differentiated 3-4-day-old fruiting bodies; 6-7-day-old fruiting bodies, before or at the beginning of spore formation, still having closed spore cases; 8-day mushrooms, heavily spore-laden, with open cases; and spores, obtained from heavily spore-laden fruiting bodies.

To obtain spores, fruiting bodies were set in a crystallizing dish covered at the top with cotton, and were left for several days in a dry warm place. The spores which fell out were fixed in 96% alcohol.

The fruiting bodies, where this was possible, were dissected into their component parts or "organs", stalk, cap, and gills, and as such were fixed in alcohol of a strength such that its final concentration was not less than 85%. The fixed material was dried by alcohol and ether and finally dried in a vacuum extractor. The substances in the alcohol-ether fraction were calculated on the basis of the dry weight of the material.

For isotope experiments, ripe 6-7-day-old fruiting bodies were used. These fruiting bodies were submerged by the base of the stalk into a water supply containing radioactive $Na_2HP^{32}O_4$ with a total radioactivity of 1 μc. The radioactivity appeared in the gills only after 3 hours' submersion of the stalk in the radioactive solution. One portion of the fruiting bodies was fixed in alcohol 5 hours after the beginning of the experiment; a second, after 15 hours. Before fixation, the fruiting bodies were dissected into stalks, caps and gills, finely crushed, and then fixed and dried in alcohol and ether as described above. Not less than 30 mushrooms on the average were taken for fractionation and chemical analysis of the fruiting bodies. In order to obtain an average sample, the material dried in alcohol and ether was carefully ground up in a mortar to a homogenous composition. The ground material was submitted to further chemical fractionation (diagram). For this we took advantage of some modifications of the method of Langen and Liss [2, 3] described in detail by us earlier [1].

As a result of such fractionation, in each case we obtained two acid-soluble fractions, the 1% TCA extract and salt extract; and three acid-insoluble, two alkaline extracts, and an extract of hot 10% $HClO_4$. Orthophosphate (P_{oph}), determined directly in the extract; 7- and 30-minute phosphorus (P_7 and P_{30}), that is, phosphorus hydrolyzed by 1 N HCl in a boiling water bath for 7 and 30 minutes respectively; total phosphorus in the extracts

(P_{tot}) were all determined in the acid-soluble fractions. The amount of polyphosphates (P_{pph}) was calculated according to the formula

$$P_{org} = P_{tot} - (P_{pph} + P_{oph}).$$

In acid-insoluble alkaline fractions the polyphosphates were calculated by the difference between the total and nucleic acid phosphorus of each fraction. In these extracts, the values obtained by the above calculation essentially corresponded to the quantity of orthophosphate, determined after 7-minute hydrolysis with 1N HCl on a boiling water bath. In fractions obtained with hot chloric acid, the content of polyphosphates was determined according to the amount of orthophosphate formed in the process of extraction. Phosphorus in all cases was determined according to the method of Berenblum and Chain as modified by Weil-Malherbe and Green [5]. Nucleic acid phosphorus was measured spectrophotometrically [6].

In the isotope experiments when it was necessary to know not only the quantity of polyphosphates found in one or another of the fractions, but also their radioactivity, they were precipitated from the extracts by barium salts at a definite pH. The acid-soluble polyphosphates in these experiments were determined in the following manner. Either from the TCA or from the salt extract after direct determination of orthophosphate, polyphosphates were precipitated by a saturated solution of $Ba(CH_3COO)_2$ at pH 4.5. Some orthophosphates, together with polyphosphates from TCA extracts, were precipitated in this way. The orthophosphate remaining in this extract was precipitated with a magnesium mixture [7]. The content of polyphosphates in the TCA extracts was calculated from the difference between the sum of the total phosphorus in the barium and magnesium precipitates and the orthophosphate phosphorus, determined directly in the extract. Because of an insignificant quantity of orthophosphate in the salt extracts the total phosphorus of the corresponding barium precipitates in these cases was calculated by the phosphorus of the polyphosphates. The acid-insoluble polyphosphates in the alkaline extracts in the isotope experiments were determined by precipitation with a saturated solution of barium salts at pH 7 [3]. The quantity of polyphosphates in these fractions was determined according to the value of orthophosphate formed by hydrolysis of barium precipitates with 1N HCl in a boiling water bath for 10 minutes. The radioactivity of the various phosphorus fractions and separate compounds was measured in a Geiger-Mueller counter. A correction for radioactive decay was introduced when calculating the radioactivity. The radioactivity of the polyphosphates was determined from the activity of the barium precipitates. In order to determine the activity of the polyphosphates in the barium precipitates obtained from the TCA extracts, a correction factor was introduced for coprecipitated orthophosphates; their activity was determined from the radioactivity of the magnesium precipitates. The radioactivity of organic phosphates in the TCA extracts was calculated by the difference in activity of the total phosphorus of this fraction and the sum of the barium and magnesium precipitates obtained from it. The radioactivity of the nucleic acid phosphorus was found by the difference between the total radioactivity of either of the acid-insoluble fractions and the activity of the polyphosphates present in it.

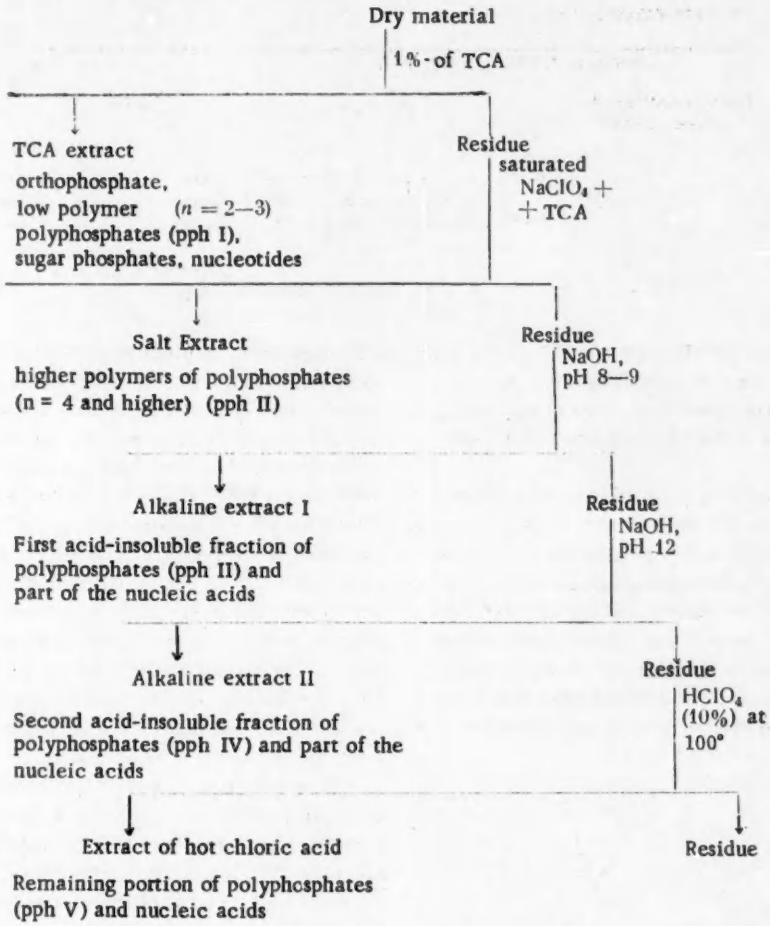
RESULTS

Data on polyphosphate content and other important phosphorus compounds in the whole fruiting body, calculated in μg P/g dry weight, are presented in Figure 1. It may be seen from this figure that the amount of orthophosphate in the whole mushroom is primarily at the same level throughout growth. The P_{tot} content, however, undergoes a marked change in the development of the fruiting body. Its variation is related basically to the changes in polyphosphates in the mushroom. It is evident that the P_{tot} and P_{pph} curves vary according to the growth and development of the fruiting body. The amount of nucleic acids in the fruiting bodies varies in a diametrically opposite direction, as expected. Examination of Fig. 1 will show that the nucleic acid curve has two maxima, of which the first, the larger, occurs during the most intensive growth of the mushroom as a whole (Table 1) and the second occurs at the beginning of spore formation. Polyphosphates, especially the acid-soluble polyphosphates, on the contrary, are present in small amounts in the mushroom during the most active synthetic period (from 1.5-2-day-old growth); and they increase again, as in the case of other mushrooms [9, 11], when the synthetic process diminishes. The most polyphosphate is accumulated between the 6th to the 7th and the 8th day of growth of the fruiting body, that is, during intensive spore formation. It is characteristic that the nucleic acid content during this period, on the contrary, is quite markedly decreased.

The inverse relationship of the curves for polyphosphates and nucleic acids is shown even more sharply by analysis of the accumulation of these compounds in separate parts of the mushroom, especially in the stalks (Fig. 2). Proceeding to an examination of these changes which various phosphorus compounds undergo in various parts of the mushroom, we first turned our attention to the stalks of the fruiting bodies, that is, to the mushroom "organ" which has direct contact with the mycelium under the ground.

Examination of Fig. 2 shows that in the beginning, that is, primarily during the first 3-4 days, a large accumulation of phosphorus occurs in the stalks of the fruiting bodies, especially in the form of P_{oph} and P_{pph}. In the next developmental period of the mushroom,

Diagram for Fractionation of Phosphorus Compounds of Mushroom Fruiting Bodies



these phosphorus compounds in the stalks sharply decrease, and at the same time, a decrease of polyphosphates is observed in the caps of the fruiting bodies also (Fig. 3). Accompanying this, as seen from Fig. 3, an extremely energetic accumulation of polyphosphates takes place in the gills of the mushroom. Consequently P_{pph} comprises about half of the total phosphorus in the gills of 6-7 day old fruiting bodies.

It may also be pointed out here that nucleic acids, like polyphosphates, are accumulated in the gills in the period between 3-4 and 6-7 days' growth. This is an exception to the general rule, and is explained, apparently, by the fact that during this time preparation for spore formation is occurring in the gills.

During the next developmental stage of the mushroom, between 6-7 and 8 days' growth, that is, during the period of active spore formation, still more intensive accumulation of polyphosphates is observed, not only in the gills, but also in the caps and stalks of the fruiting bodies. Simultaneously a pronounced fall in nucleic acid content in all parts of the fruiting body is observed. It is also shown that during this period an increasing accumulation of acid-soluble organic phosphorus compounds occurs in all parts of the mushroom. It is not impossible that this is related to partial hydrolysis of nucleic acids apparently occurring at this time.

It may be further observed that the same data of Figs. 2 and 3 indicate changes in the total quantity of polyphosphate in separate parts of the fruiting body according to its development. However, by the fractionation diagrammed above, all polyphosphates in the mushroom were successfully separated into five different fractions. Data on the content of polyphosphates and other phosphorus compounds in these fractions calculated in μg P/g dry weight are presented in Table 2. Examination of the data presented in this table leads to the conclusion that in all parts of the fruiting body as in the whole mushroom [1] throughout its development, the main portion of the polyphosphates are found in the

TABLE 1. Change of Dry Weight of the Fruiting Body of the Mushroom and its Separate Parts According to its Growth and Development. Dry weight expressed in g.

Growth of fruiting body, days					
Undifferentiated 1.5-day-old	2	3 4	6 7	8	"Organ"
0,00056	0,0704	0,139	0,252	0,438	Gills
		0,351	0,687	0,985	Caps
	0,0492	0,227	0,371	0,384	Stalks
0,00056	0,1196	0,717	1,310	1,807	Fruiting body as a whole

acid-soluble fractions. In all cases 85-90% of the acid-soluble fraction consists of polyphosphates. It is noteworthy that polyphosphates are present in both acid-soluble fractions – the TCA extract and the salt fraction.

As we showed previously [1], only pyro-tripoly-, and trimetaphosphates are extracted from the mushrooms by 1% TCA, while more highly polymerized polyphosphates (tetrapolyphosphates and higher) are found in the salt extract. The present data prove that the acid soluble polyphosphates in all parts of the mushroom are distributed essentially equally by extraction between both fractions. One of the few exceptions to this is the acid-soluble polyphosphate ofgills of the spore-bearing 8-day-old mushrooms, in which the accumulated polyphosphates are comparatively more highly polymerized and were largely extracted by a saturated solution of sodium perchlorate. A large part of the acid-insoluble polyphosphates in almost all cases is extracted by weak alkali at pH 8-9. The polyphosphates in mushrooms remaining after this extraction are not completely extracted in stronger alkali at pH 12, as for example with yeasts [3]. A very small part of the polyphosphates, apparently the most firmly bound with the organic components of the cell, are extracted successfully only by treating the precipitate with hot chloric acid. Let us note, by the way, that nucleic acids also are found in all acid-insoluble fractions, and usually over half of them are extracted in weak alkali.

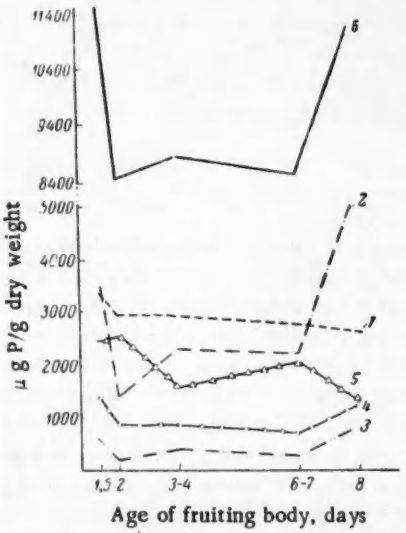


Fig. 1. Change in content of the principal phosphorus compounds in the process of development of fruiting bodies of the mushroom (in the whole mushroom). Data calculated in μgP/g dry weight of the whole fruiting body. A) μg P/g dry weight; B) Age fruiting body, days; 1) P_{oph}; 2) $P_{a\text{-}sol\ pph}$; 3) $P_{a\ insol\ pph}$; 4) $P_{org\ TCA\ extract}$; 5) P_{na}; 6) P_{tot}.

However, data presented in Table 2 and Figs. 1-3 calculated on the one gram dry weight basis for each separate part of the fruiting body did not allow us to solve the problem of the relationship of these parts to the metabolism of polyphosphates and other phosphorus compounds. By such calculations it was impossible to establish in what degree and by what type of compound the transport of phosphorus from one part of the mushroom to another took place. Comparison of data calculated in this way is especially difficult because dry weights of the various parts of the mushroom decrease in a different degree, as can be seen from the data of Table 1.

Consequently, by using the data of Table 1 we calculated the amounts of both total phosphorus and phosphorus of all compounds which interested us, in each separate part of the fruiting body in all stages of development. The values obtained for the various parts of the mushroom at a certain age can be added together so that data for the fruiting body as a whole may be obtained. Results of similar calculations are presented in Table 3. An uninterrupted accumulation of phosphorus in the mushroom fruiting bodies during their growth process is observed from this table. This process is especially energetic between the 1.5- and 2-day-old growth, when P_{tot} in the mushroom is increased more than 150 times. It is true that the mass of the mushroom is increased 200 times during this period,

TABLE 2. Change in the Content of Polyphosphates and some other Phosphorus Compounds in the Process of Development of Fruiting Bodies of Mushrooms (in μg P/g Dry Weight)

Age, days	Fruiting body	Fraction																								Sum
		1% TCA at 0°				$NaClO_4$ at 0°				NaOH at pH 8-9; 0°			NaOH at pH 12 at 0°			10% $HClO_4$ at 100°			P_{tot} in material	Sum P_a sol pph	Sum P_a insol pph	Sum P_{na}
		P_{oph}	P_{pph}	P_{org}	P_{tot}	P_{oph}	P_{pph}	P_{org}	P_{tot}	P_{pph}	P_{na}	P_{tot}	P_{pph}	P_{na}	P_{tot}	P_{pph}	P_{na}	P_{tot}				
1,5	whole fruiting body	3310	1690	1350	6350	143	1757	200	2100	310	1200	1510	113	295	408	176	912	1080	11 440	3447	589	2407
2	S	2840	680	720	4240	28	658	138	824	205	1225	1430	22	244	266	86	906	1060	7830	1338	313	2375
	C + G	2840	660	960	4460	19	771	960	1750	25	1395	1420	19	338	357	64	813	838	8810	1431	108	2546
3—4	S	4390	1640	1400	7430	0	1160	1200	2360	520	945	1465	225	291	516	27	432	544	12 360	2800	872	1668
	C	2560	830	530	3920	219	706	430	1355	35	710	745	27	215	242	12	201	239	6480	1536	74	1126
	G	1170	1130	780	3080	0	2000	630	2630	440	1350	1790	70	470	540	42	680	816	8860	3130	552	2500
6—7	S	3140	840	1090	5070	0	1030	590	1620	170	1380	1550	36	282	318	32	421	497	9090	1870	238	2083
	C	2680	280	500	3460	77	613	362	1052	68	585	653	56	180	236	11	269	318	5700	893	135	1034
	G	1980	1830	760	4570	0	4230	870	5100	80	570	2650	204	710	914	312	1258	1645	14 860	6060	596	4538
8	S	3750	1770	1105	6625	65	1615	200	1880	379	595	974	0	181	172	55	246	300	9960	3385	634	1022
	C	2580	2840	1280	6700	0	1978	0	1978	458	452	910	88	114	202	25	214	242	10 040	4558	571	780
	G	1540	1890	1180	4610	0	4700	1400	6100	1140	1800	2940	189	373	562	42	630	708	15 060	6590	1371	2803

Note. In this and the following tables: S = stalk, C = cap and G = gills; P_a sol pph = acid-soluble polyphosphate phosphorus; P_a insol pph = = acid-insoluble polyphosphate phosphorus.

which results in some decrease in total phosphorus in the whole fruiting body as we have seen in calculations on the one gram dry weight basis (Fig. 1). Beginning with the 2-day-old growth, the process of phosphorus accumulation in the fruiting body, although it is decreased somewhat, nevertheless continues to proceed quite energetically. As a result of this, total phosphorus in 8-day-old mushrooms is increased 3,000-fold while during that time polyphosphates increase 5,000 times and nucleic acid, only 1500 times. By examination of the data in Table 3 it is also evident that there is very little decrease in P_{tot} and especially P_{oph} in the stalks beginning with 3-4-day-old mushrooms. However, a very large quantity of phosphorus is accumulated in the caps and gills during this time. It is not impossible that phosphorus transport from the stalks to the caps and gills may be accomplished along with other phosphorus compounds, and in the form of low-polymer polyphosphates. This is borne out particularly by the following fact. The highest amount of low-polymer polyphosphates is found in the stalks in 3-4-day-old mushrooms, while just before the beginning of spore formation, when the mushrooms are 6-7 days old, the polyphosphate maximum occurs in gills. Thus the overall quantity of low-polymer polyphosphates in the stalks and caps of 6-7-day-old mushrooms is markedly lower in comparison with those 3-4 days old. Attention is further drawn also to data from Table 3 indicating that in the period between 6-7 and 8 days' growth, nucleic acids in the stalk are sharply decreased. Nucleic acid remains primarily at the same level in the cap and gills. These data again verify the assumption made above that during the most intensive spore formation in the fruiting body, nucleic acid moities are split up into mononucleotides which are capable of being transported from one part of the mushroom to the other.

All the considerations given above require verification and refinement. In addition, the experimental material examined above does not give a total answer to a series of essential questions. For example, whether polyphosphates ave synthesized in the fruiting bodies themselves or whether they are carried there from the mycelium is a problem that has not been considered. On the other hand, the form of the compound by which the transport of phosphorus into the fruiting body chiefly occurs also remains obscure. We tried to answer these and other questions by the use of radioactive P^{32}.

It was found in the first work with radioactive P^{32} that the presence of the stalk end of the fruiting body was necessary for absorption of phosphorus from the water solution, that is, the portion of the stalk which is directly fastened to the mycelium or spawn. If this part of the mushroom was cut away, then the transport of marked phosphate into the fruiting bodies was prevented. On the other hand, when the entire fruiting body was incubated in an isotope solution, P^{32} could be

found within 3 hours in all parts of the mushroom.

As the data of Table 4 indicate, 5 hours after the beginning of the experiment, P^{32} was present not only in the orthophosphate fraction, but also in other phosphorus compounds. Especially high specific activity aside from that of orthophosphate was found in the acid-soluble organic fractions in all parts of the mushroom. The specific activity of this phosphorus fraction in caps, both at 5 and 15 hours' incubation, was significantly higher than the orthophosphate specific activity in the caps. Consequently, it could be concluded from this fact that transport of phosphorus from the stalks to the caps was accomplished generally not in the form of orthophosphate but as some type of organic phosphorus compound. In the gills, however, orthophosphate had the highest specific activity. This can be explained on the basis that introduction of phosphorus in the gills occurs mainly in the form of orthophosphate. From these data, we must conclude that there are at least two different pathways of phosphorus transport from one part of the mushroom fruiting body to the other. Via one route – from stalk to cap – phosphates take the form of some type of organic compound, thereby serving as a main source of phosphate for other synthetic processes. On the other hand, transport of phosphorus from stalks to gills apparently bypasses the cap and phosphorus acts directly as othophosphate. This is borne out by the fact that after 15 hours incubation, orthophosphate specific activity in the gills is higher than the specific activity of all phosphorus compounds in the cap of the mushroom. Metabolism of phosphorus compounds must also undoubtedly exist between the cap of the fruiting body and its gills. Isotope data, particularly, indicate the possibility that low polymer polyphosphates participate in the transport of phosphorus from caps to gills. As shown in Table 4, during the period between 5 and 15 hours' incubation the low-polymer polyphosphate fraction is quite markedly decreased in the fruiting body caps with a constant amount of orthophosphate, and in gills this fraction is increased. In addition, the specific activity of both parts of the

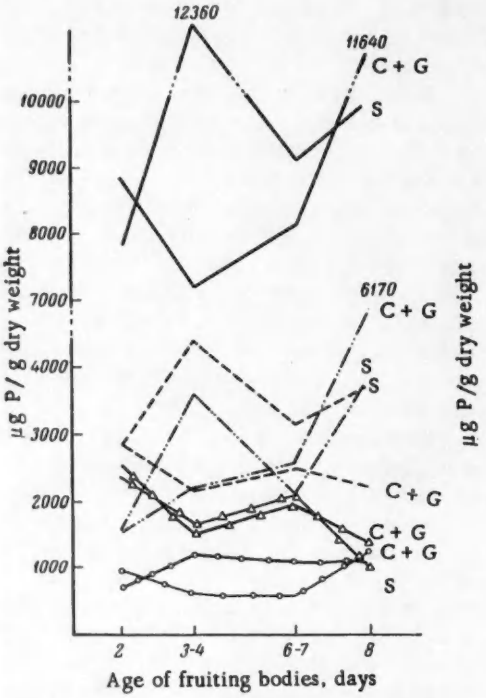


Fig. 2. Change in the content of the principal phosphorus compounds in the process of development of fruiting bodies of the mushroom (separated into stalks, and caps and gills, combined). The data are calculated in µg P/g dry weight of the corresponding stalks and sum of the caps and gills. Designations the same as in Fig. 1, except: dotted line with two points – P sum of acid soluble and acid insoluble pph; s = stalks, C + G = gills.

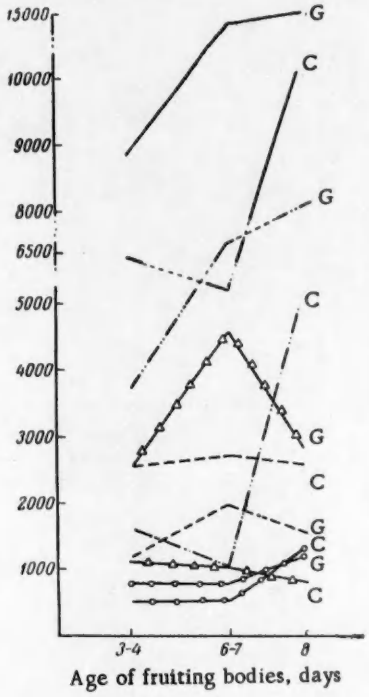


Fig. 3. Change in content of the principal phosphorus compounds in the process of development of the fruiting bodies of the mushroom (separated into caps and gills). Data are calculated in µg P/g dry weight of the corresponding caps and gills. Designations the same as in Fig. 2.

TABLE 3. Change in the Content of Polyphosphates and some Other Phosphorus Compounds in the Process of Development of Fruiting Bodies of the Mushroom (in μg P in a single Fruiting Body)

Form of phosphorus	Time of growth, days															
	1,5	2			3–4				6–7				8			
	Entire fruiting body		S	C + G	Entire fruiting body	S	C	G	Entire fruiting body	S	C	G	Entire fruiting body	S	C	G
P_{oph}	1,855	340,0	140,0	200,0	2058	998	897	163	3504	1164	1841	499	4655	1440	2540	675
$P_{pph\ I}$	0,946	79,9	33,4	46,5	829	381	291	157	966	312	192	462	4306	679	2800	827
$P_{pph\ II}$	0,984	86,7	32,4	54,3	788	263	247	278	1869	382	421	1066	4625	620	1950	2055
$P_{a\ sol\ pph}$	1,930	166,6	65,8	100,8	1617	644	538	435	2835	694	613	1528	8931	1299	4750	2882
P_{org}	0,756	102,9	35,4	67,5	607	313	186	108	938	404	343	191	2202	425	1260	517
$P_{a\ insol\ pph}$	0,335	22,9	15,3	7,6	278	175	26	77	332	88	94	150	1330	167	562	601
P_{na}	1,347	296,2	117,0	179,2	1123	378	395	350	2628	772	712	1144	2382	393	768	1221
P_{tot}	6,410	1006,0	385,0	621,0	6310	2800	2280	1230	11 030	3360	3920	3750	20 380	3820	9990	6570
Sum of P_{pph}:	2,265	189,5	81,1	108,4	1895	819	564	512	3167	782	707	1678	10 261	1466	5312	3483

TABLE 4. Content and Radioactivity of Polyphosphates and some other Phosphorus Compounds in Fruiting Bodies of the Mushroom after 5- and 15-Hour Incubation in a Solution Containing P^{32}. Data in μg P/g dry weight. Radioactivity in impulses / min.

Time of incubation of mushrooms in P^{32}, hours	Fruiting body	Measurement	1% TCA at 0°				$NaClO_4$ at 0°		NaOH at 0°, pH 8-9			NaOH at 0°, pH 12			10% $HClO_4$ at 100°	P_{tot} in fractionation	P alcohol-ether fraction
			P_{oph}	P_{pph}	P_{org}	P_{tot}	P_{pph}	P_{tot}	P_{pph}	P_{na}	P_{tot}	P_{pph}	P_{na}	P_{tot}	P_{tot}		
5	S	P	8000	0	3440	11 440	1570	2620	246	838	1310	126	188	331	859	16 800	5920
		imp / min	1 0330	—	43 900	14 7200	6460	11 890	998	502	1500	899	41	940	1500	162800	46 700
		imp / min / P	12,92	—	12,76	12,88	4,11	4,54	4,06	0 60	1,14	7,13	0,22	2,84	1,75	9,69	7,89
	C	P	5990	1260	1520	8770	1220	1400	98	651	958	25	363	256	342	11 833	2410
		imp / min	4350	440	3650	8440	468	480	48	91	139	24	38	14	32	9129	1350
		imp / min / P	0,727	0,349	2,400	0,962	0,384	0,343	0,490	0,140	0,145	0,968	0,105	0,055	0,034	0,771	0,560
	G	P	2230	720	2040	4990	6330	6340	619	798	1570	240	385	732	1660	13900	4780
		imp / min	4190	200	2840	7230	1760	1760	314	96	410	210	11	221	340	9620	4230
		imp / min / P	1,880	0,278	1,372	1,449	0,278	0,277	0,507	0,120	0,261	0,875	0,029	0,302	0,205	0,692	0,885
15	S	P	6540	1850	2380	10 770	3560	7020	246	585	1060	108	173	254	790	19 820	6880
		imp / min	74 050	18 540	32 410	12 5000	28 600	39 750	3380	1510	5700	1525	250	1775	3490	17 8000	51 600
		imp / min / P	11,32	10,02	13,62	11,61	8,03	5,66	13,74	2,58	5,38	14,12	1,44	6,99	4,42	8,99	7,50
	C	P	5803	790	2180	8770	1360	1840	164	636	1190	43	285	383	332	12 515	2220
		imp / min	4590	670	4550	9810	1210	1210	156	96	252	42	10	52	57	11 232	690
		imp / min / P	0,791	0,848	2,087	1,118	0,890	0,658	0,929	0,150	0,212	0,977	0,035	0,136	0,175	0,897	0,311
	G	P	1780	910	1880	4570	7750	7850	696	585	1590	92	229	390	1300	15600	5410
		imp / min	6620	760	5920	13 300	9990	10 100	1950	480	2430	265	126	391	1080	26 650	15 710
		imp / min / P	3,725	0,835	3,149	2,910	1,289	1,286	2,802	0,820	1,528	2,880	0,550	1,003	0,830	1,708	2,904

TABLE 5. Content of Polyphosphates and other Phosphorus Compounds in Spores and Heavily Spore-Laden Gills of Fruiting Bodies of the Mushroom

Forms of phosphorus	Fraction																		Sum		
	1% TCA at 0°				$NaClO_4$ at 0°				NaOH at 0°, pH 8-9			NaOH at 0°			10% $HClO_4$ at 100°			P_{tot} in material	P_a sol pph	P_a insol pph	P_{na}
	P_{oph}	P_{pph}	P_{org}	P_{tot}	P_{oph}	P_{pph}	P_{org}	P_{tot}	P_{pph}	P_{na}	P_{tot}	P_{pph}	P_{na}	P_{tot}	P_{pph}	P_{na}	P_{tot}				
Spores •	680	236	654	1570	18	969	511	1498	440	90	828	92	28	220	238	750	918	5034	1205	770	868
Spore-bearing gills •	3360	9890	2430	15 680	52	3840	958	4850	450	362	1080	71	102	282	85	444	558	22 450	13 730	606	908
Spores †	0,99	0,34	0,95	2,28	0,03	1,40	0,74	2,17	0,64	0,13	1,20	0,13	0,04	0,32	0,34	1,09	1,33	7,30	1,74	1,11	1,26
Spore-bearing gills †	2604	7666	1884	12 154	40	2977	743	3759	349	280	837	55	79	219	66	344	433	17 402	10 643	470	703

• Calculated in μg P/g dry weight

† Calculated in μg P per fruiting body

mushroom during this time is practically constant. Thus, data obtained with P^{32} once again verify the possibility that low-polymer polyphosphates participate in the movement of phosphorus from one part of the fruiting body to the other. Also, the use of the isotope method allowed us to establish the ability of all parts of the fruiting body to synthesize polyphosphates independently. Actually, examination of Table 4 shows that the acid-insoluble fraction has the highest specific activity of all polyphosphate fractions, particularly those which are extracted with comparatively strong alkali at pH 12. As is known [2, 3, 9, 10, 12, 13], the synthesis of polyphosphates occurs particularly in the acid-insoluble fraction, as has recently been shown for yeasts [2, 3], in the fraction extracted by alkali at pH 12. But it must be remembered here that another fraction of polyphosphates exists in the mushroom fruiting bodies (pph V) which can be extracted only by hot chloric acid. It is completely possible that primary synthesis of polyphosphates occurs in this case. This is indirectly indicated (Table 4) by quite high specific activity values for P_{tot} of this fraction, sometimes even exceeding the corresponding values obtained for total phosphorus in the second alkaline fraction (for example, in the mushroom caps). In any case, the isotope data prove that since acid-soluble polyphosphates exist in mushroom fruiting bodies in such large quantity, they are not brought in from the spawn, but are synthesized directly in all parts. As seen from the data of Table 4, this synthesis is carried out starting with orthophosphate through the intermediate formation of acid-soluble organic phosphorus compound.

In this way, the total picture of synthesis and stepwise depolymerization of polyphosphates in mushrooms, corresponding to literature data for other organisms [2-4, 9, 10-15], can be represented in the following scheme.

Orthophosphate→acid-soluble organic phosphorus compounds→[pph V]→pph IV→pph III→pph II→pph I→orthophosphate. By analysis of all the above data it can be concluded that this process results in the formation principally of low-polymer polyphosphate fractions pph II and pph I in the mushroom fruiting bodies. Accumulation of these polyphosphate fractions is increased especially with development of the mushroom to the spore-formation state, particularly in the spore-forming organ, the gills. It would be interesting to clarify the role of the low-polymer polyphosphates in the process of spore formation. Are they a form of transport in the spores and a deposit for large quantities of phosphorus and energy, or do they participate themselves in the act of spore formation? In order to clear up this question it was important to know what part of the polyphosphates, contained in the spore-forming gills, can be found in the spores thrown off by them. For this purpose, we analyzed the phosphorus compounds con-

tained in the spores and spore-bearing gills after the spores were shed. The results obtained expressed in μg P/g dry weight and on a single fruiting body are presented in Table 5.

Examination of the data in Table 5 shows, first of all, that all the phosphorus fractions found in fruiting bodies also exist in spores. However, distribution of phosphorus between separate phosphorus fractions sharply differentiates the spores both from the whole fruiting bodies [1] and from the spore-bearing gills. For example, spores contain a very large number of acid-insoluble polyphosphates, about 40% of the total polyphosphate content. Besides, a large part of the nucleic acids in spores, unlike the fruiting bodies, are very closely bound to other cellular components and are not extracted even by comparatively strong alkali.

It is noteworthy that in spite of the large difference of total phosphorus content in spores and spore-bearing gills (in spores, 4-5 times less) the sum of nucleic acids in both cases is almost the same. Polyphosphates in spore-bearing gills are seven times higher than in spores; this difference is entirely at the expense of acid-soluble and especially low-polymer polyphosphates. As chromatographic analysis showed, polyphosphates are present in heavily spore-laden gills mainly in the form of pyro, tri-, poly-, and trimetaphosphates. As the same time, as seen in Table 5, the amount of low-molecular-weight polyphosphate in the spores is not very large. The thought then occurs that the accumulation of large amounts of low-polymer polyphosphates in gills during the spore-forming process was not connected, apparently, with their further transport in spores. Calculation of the data obtained in μg P / mushroom (Table 5) shows that only one one-hundredth as much polyphosphate accumulates in spore-bearing gills as occurs in the spores given off. This fact also is evidence against the role of polyphosphates as participants in the transport of phosphorus from the fruiting body to the spores. It is preferable to consider that their accumulation in the mushroom in such large quantity was due to spore formation itself.

It may be supposed that the energy of polyphosphates in mushroom fruiting bodies, which arises from hydrolysis of the most highly polymerized to lower polymerized fragments is used for the process of scattering spores.

SUMMARY

A large quantity of polyphosphates are found in all parts of the fruiting body of the double-spored mushroom. The total quantity of polyphosphates in the fruiting body is markedly increased during its growth and development, attaining a maximum value in the spore-bearing mushrooms. In all parts of the mushroom, most of the polyphosphates are in the form of comparatively low-polymer fragments and are found in the acid-soluble fractions.

Polyphosphates are formed in the fruiting body itself, equally in the stalk, cap and gills of the mushroom. The biosynthesis of polyphosphates progresses from orthophosphate through the intermediate formation of some type of acid-soluble phosphorus compounds, resulting in the formation of essentially acid-insoluble phosphate compounds.

A close connection was established between the synthetic process of polyphosphates and the formation of nucleic acids. A competitive relationship exists between these two processes in the mushroom fruiting body.

Accumulation of a large quantity of polyphosphates in the mushroom fruiting body is connected, probably, with their active participation in the process of spore formation. An increase in this process results in a strong intensification of polyphosphate synthesis in all parts of the mushroom and their accumulation in the spore-forming organs – the gills. Thus, it was shown that low-polymer fragments of polyphosphates can be transported without hydrolysis to orthophosphates from one part of the fruiting body to another.

However, by means of P^{32} it was established that the main form of phosphorus transport inside the fruiting body was orthophosphate and some acid-soluble organic compound. It was thus made clear that there exist at least two different and independent pathways of phosphorus transport in fruiting bodies. One pathway from stalk to cap generally takes place by means of some type of acid-soluble organic phosphorus compound. The other pathway from stalk to gills bypasses the cap, and occurs chiefly in the form of orthophosphate.

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THE MOLECULAR WEIGHT OF POLYMYXIN M

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Many antibiotics have molecular weights in the range 500-2000. It is well known that accurate molecular weight determinations for these compounds present great difficulties. In recent years Craig et al.[1] have proposed a new method of partial substitution which was successfully employed in the study of gramicidin S, tyrocidin A [2], polypeptin [3], bacitracin A [4], polymyxin B [5], and insulin [6].

Being faced with the necessity of determining the molecular weight of polymyxin M, which was isolated in the USSR, we decided to use this method.

It has previously been shown [7] that polymyxin M is a cyclopolypeptide consisting of one leucine residue, three threonine residues, and six α, γ-diaminobutyric acid residues. In addition there is present a fatty acid similar to, and possibly identical with, 6-methyl octanoic acid. The only functional groups in polymyxin M are the amino groups of α, γ-diaminobutyric acid. The molecular weight of polymyxin M is equal to the product of its equivalent weight and the number of amino groups present. We have found the equivalent weight, i.e., that part of the molecular weight associated with one amino group, to be 237. In order to determine the number of free amino groups in the molecule we used the reaction of partial dinitrophenylation.

Upon reacting the antibiotic with a small amount of dinitrofluorobenzene, an extremely complex mixture of products of partial substitution was obtained. The composition of this mixture depends on the number of free amino groups in the antibiotic. If there are three free amino groups, then there will be three reaction products: mono-dinitrophenyl (DNP)-polymyxin M, di-DNP-polymyxin M, and tri-DNP-polymyxin M. If there are five free amino groups, there will be five reaction products, etc. (We do not take into account isomers, which differ only in the position of the dinitrophenyl groups, inasmuch as they are not separated by paper electrophoresis).

The mixture of reaction products, which corresponds to various degrees of substitution, must then be separated into its components. Such separation may be achieved by paper electrophoresis [8]. The method of separation used here is based on the fact that during successive substitutions of the amino groups in the antibiotic molecule by a dinitrophenyl group or by any axyl residue (for example, the carbobenzoxy group) there is a stepwise decrease in molecular charge which results in a corresponding reduction of electrophoretic mobility.

Thus, in the determination of the number of free amino groups it is necessary, first, to select reaction conditions which will ensure the recovery of all possible substitution derivatives, and, second, to select conditions of electrophoresis which will give the most complete separation of the reaction products.

EXPERIMENTAL METHODS

Starting materials. Polymyxin M sulfate (activity 10,000 units/mg) was obtained at the All-Union Research Institute of Antibiotics using ion-exchange resins (material electrophoretically homogeneous). Imported preparations of polymyxin B sulfate and gramicidin S hydrochloride which had been purified by repeated crystallization were also used.

Synthesis of DNP-polymyxin M. To a solution of 0.1 g (0.07 mole) polymyxin M sulfate in 1 ml of water and 0.6 ml of acetone were added 0.2 ml (1.52 mmole) triethylamine and 0.25 g (1.3 mmole) 2,4-dinitrofluorobenzene (DNFB) in 1 ml of acetone. The mixture was shaken one hour at 20° and then allowed to stand 2.5 hours at 37°, after which it was evaporated under a vacuum at 25°. To remove the triethylamine, the dry residue was three times taken up in water and then dried under a vacuum, being finally dissolved in a minimal amount of acetone. DNP-polymyxin was precipitated from the acetone solution with saturated ammonium sulfate. The precipitate was carefully washed with distilled water, dried in air, and extracted with ether. The resulting product was a yellow amorphous material (0.102 g). The purity of the DNP-polymyxin obtained was controlled electrophoretically. DNP-polymyxin M is insoluble in water, ethanol, butanol, ether, benzene, chloroform, carbon tetrachloride, and dioxane; it is slightly soluble in acetone and glacial acetic acid and soluble in 85% formic acid. The melting point is

214-217° (with decomposition). A solution of DNP-polymyxin M in 85% formic acid has a maximum absorption at 355 mμ.

Determination of equivalent weight of polymyxin M. Five solutions of DNP-polymyxin M in 85% formic acid ranging in concentration from 5.28 to 13.45 μg/ml were prepared. Their optical density was measured at 355 mμ on an SF-4 spectrophotometer using a 1-cm quartz cuvette. In the calculation of the equivalent weight, we used the molecular extinction coefficient for the dinitrophenyl group determined by Battersby and Craig [2], 15,000. The equivalent weight of the free base of polymyxin M is 237 ± 5.

Partial dinitrophenylation of polymyxin M in the presence of the anion-exchange resin ÉDÉ-10P. About 100 mg of the resin in the OH form and 1 ml of acetone were aded to a solution of 10 mg (0.035 milliequivalents) polymyxin M sulfate in 1 ml of water. The mixture was shaken for 2 minutes, and then 0.33 ml of a solution of DNFB in acetone (concentration 100 mg/ml, 0.017 mmole) was added. The mixture was shaken two hours at room temperature and then allowed to stand 2.5 hours at 37°. The solution was evaporated under a vacuum at 25°, the dry residue taken up in 1 ml of 85% formic acid, and the resin removed by centrifugation. The yellow solution obtained was spotted on chromatographic paper for electrophoresis. In the experiment described, half an equivalent of dinitrofluorobenzene was reacted with one equivalent of polymyxin M. Experiments were performed under the same conditions in which 1/8, 1/4, 1, and 2 equivalents of dinitrofluorobenzene were reacted with an equivalent of polymyxin M.

Paper electrophoresis of the products of partial dinitrophenylation. Electrophoresis was carried out in an apparatus similar to that described by Durrum [9]. The chromatographic paper used was of the slow filtration type from the Volodar plant in Leningrad. Paper strips were 34 cm in length, and separation was carried out at 220 volts. The solution employed was composed of 85% HCOOH, glacial acetic acid, and water (28 : 20 : 52 by volume); current strength was 0.03-0.05 milliamps/cm; electrophoresis was carried out for 6.4 hours. Location of the DNP-derivatives on the chromatogram was not difficult, since they were yellow in color. In addition, we routinely examined the strips in a Brumberg ultrachemiscope. Free polymyxin was located by spraying with 0.5% ninhydrin. Since they contained free amino groups, the partially substituted DNP-derivatives also reacted with hir.hydrin to form colored products. The results of electrophoretic separation of the products of partial dinitrophenylation of polymyxin are presented in Table 1.

Partial dinitrophenylation of the free base of polymyxin M. The free base was obtained by passing an aqueous solution of polymyxin M sulfate through a column of ÉDÉ-10P in the OH form and then evaporating the filtrate under a vacuum. To 10 mg of the product dissolved in 1 ml of water was added 2.3 ml of a solution of DNFB in acetone (10 mg/ml). The mixture was shaken 2 hours at 20° and then kept at 37° for 2.5 hours. It was then evaporated under a vacuum at 25°. The dry residue was dissolved in 0.8 ml 85% HCOOH and subjected to electrophoresis. In a parallel experiment, 0.1 ml of a solution of DNFB in acetone was added to the same amount of polymyxin.

TABLE 1. The Position on an Electrophoresis Chromatogram of Products of Partial Dinitrophenylation of Polymyxin M in the Presence of the Exchange Resin ÉDÉ-10P

Equivalent ratios of polymyxin M & DNFB	Distance of spots from the origin, cm (movement toward the cathode)					
1 : 2	0	1,5	3,0			
1 : 1	0	1,5	3,0	4,4	5,8	
1 : 1/2	0	1,5	3,0	4,4	5,8	7,3*
1 : 1/4			3,0	4,4	5,8	7,4*
1 : 1/8			3,0	4,4	5,8	7,3*

*Spots were sprayed with ninhydrin only. Polymyxin M moved 7.3 cm.

The solution used in electrophoresis was composed of 85% HCOOH, glacial acetic acid, and water (20 : 24 : 56 by volume); electrophoresis was carried out for 7 hours; in other respects the experiment was the same as that described above. The spots were located on the chromatogram as follows:

Equivalent ratios of polymyxin M and DNFB	Distance of spots from the origin, cm (movement toward the cathode)					
1 : 3	0	1.7	3.4	5.2	7.1	8.7*
1 : 1/8			3.4	5.2	7.1	8.7*

*Chromatogram was sprayed with ninhydrin only. Polymyxin M moved 8.7 cm.

Partial dinitrophenylation of the free base of polymyxin M on paper. Fifty mg of the exchange resin ÉDÉ-10P in the OH form was added to a solution of 10 mg of polymyxin M sulfate in 1 ml of water. The mixture was shaken 20 minutes and the resin centrifuged off. The resulting solution was then applied with a micropipette to a strip of chromatographic paper 36 cm long, in three spots (2, 4 and 6 μliters per spot) at a distance 1 cm from the median line. The areas to which polymyxin M had been applied were then treated with a 20 mg/ml solution of DNFB in acetone (~0.6 mg DNFB per spot) in a stream of warm air. The DNFB solution was first applied to the tip of a glass rod with the micropipette. Upon contact of the rod with the paper, the solution runs off toward the center of the polymyxin spot. This method of DNFB application has

the advantage that the material being treated is not spread more widely on the paper but is instead concentrated further. Following treatment of the free base of polymyxin M with DNFB, the paper strip was kept at 37° for 25 minutes, after which it was sprayed carefully, beginning from the ends, with a mixture of 85% HCOOH, glacial acetic acid, and water (28 : 20: 52). The moist paper strip was then placed in the electrophoresis apparatus and a current was applied. Duration of the experiment was 5 hours. Upon completion of the experiment, five yellow spots, corresponding to various DNP-derivatives of polymyxin M, were found on the paper. One of them was at the origin, and the others had moved 1.5, 3.0, 4.5, and 5.9 cm toward the cathode. Upon treatment with ninhydrin, a spot corresponding to unsubstituted polymyxin, which had moved 7.4 cm, was visible.

Experiments with carbobenzoxy-polymyxin M. To a solution of 50 mg polymyxin M sulfate in 10 ml 0.05N sodium tetraborate chilled to 0° was added 0.12 ml carbobenzoxychloride in 5 ml 0.1N sodium tetraborate. After being shaken 40 minutes at 20°, the mixture was evaporated under a vacuum. The dry residue was carefully washed with water and dried over H_2SO_4. Forty mg of carbobenzoxy-polymyxin M (m. p. 220-222° with decomposition) was obtained. In contrast to the original polymyxin M, this substance does not give a ninhydrin reaction, is insoluble in water, and remains at the origin during electrophoresis.

Removal of the carbobenzoxy group was carried out in the following manner. A stream of hydrogen was passed through a solution of 20 mg carbobenzoxy-polymyxin M in 2 ml 85% HCOOH containing 20 mg palladium black, the mixture being shaken in the process. At intervals samples were withdrawn with a 4-microliter pipette and applied to a strip of chromatographic paper for electrophoresis. Electrophoresis was carried out in 30% acetic acid at 600 volts for 3.5 hours. Results of electrophoretic separation are presented in Table 2.

The solution obtained after hydrogenolysis, which contained free polymyxin, was filtered and evaporated under a vacuum. Determination of the anti-bacterial activity of the regenerated polymyxin M* showed that in this respect it is the same as the original antibiotic.

Experiments with polymyxin B. Determination of the equivalent weight of polymyxin B was carried out in the same manner as with polymyxin M. It was found to be 252 ± 10.

Partial dinitrophenylation of polymyxin B in the presence of the resin ÉDÉ-10P was carried out as for polymyxin M. Electrophoretic separation of the reaction products was performed at 220 volts for 6.5 hours using a mixture of 85% HCOOH, acetic acid, and water (28 : 20 : 52). Five yellow DNP-derivatives were obtained of which one remained at the origin and

TABLE 2. The position on an Electrophoresis Chromatogram of Products Formed During the Removal of the Carbobenzoxy Group from Carbobenzoxy-Polymyxin M

Period of hydrogenation, min.	Distance of spots from the origin, cm (movement toward the cathode)					
0	0					
5	0	1,8	4,6	7,0	8,6	9,6
15				7,0	8,6	9,6
35					8,6	9,6
75					8,6	9,6
150						9,6
360						9,6

*The chromatogram was sprayed with chlorine and benzidine [10]. Under these conditions, polymyxin M moved 9.6 cm toward the cathode.

the others migrated 1.4, 2.9, 4.2, and 5.6 cm toward the cathode. Unsubstituted polymyxin B was also found 6.9 cm from the origin. When the products of partial dinitrophenylation of polymyxins M and B were subjected to electrophoresis at the same time, the corresponding derivatives migrated toward the cathode at identical rates. Data on relative mobilities of DNP-derivatives of polymyxin B are given in Table 3.

Partial dinitrophenylation of polymyxin B on paper was carried out as described above. Upon electrophoretic separation of the reaction products in the mixture 85% HCOOH – CH_3COOH – water (28 : 20 : 52), five derivatives were found. These migrated toward the cathode at 220 volts in 6.75 hours 0, 1.5, 3.0, 4.4, and 5.8 cm respectively. Unsubstituted polymyxin B migrated 7.3 cm.

Experiments with gramicidin S. Determination of the equivalent weight according to the method described gave a value of 554 ± 5. Equivalent weight calculated from the amino acid composition is 570.

Partial dinitrophenylation of gramicidin was carried out in alcoholic medium in the presence of the resin ÉDÉ-10P in the OH form. One equivalent of gramicidin S was allowed to react with ½, 1, and 2 equivalents of DNFB. Experimental conditions were the same as for polymyxin M. Reaction products were subjected to electrophoretic separation at 220 volts for 5 hours using 30% CH_3COOH (see tabulated data on following page).

DISCUSSION

The partial dinitrophenylation reaction was utilized to determine the number of free amino groups in polymyxin M. Recovery of the whole gamut of possible derivatives was ensured by the fact that the antibiotic was allowed to react with various amounts of DNFB in

*Determination was made by T. P. Udalova, an associate in the Laboratory of Antibiotics, Soil Biology Faculty, Moscow State University.

TABLE 3. Relative Electrophoretic Mobilities of DNP-Deriatives of Polymyxin B

Derivatives	Observed	Calculated for		
		4 NH_2-groups	5 NH_2-groups	6 NH_2-groups
Unsubstituted polymyxin B	1.0	1.0	1.0	1.0
Mono-DNP	0.81	0.75	0.80	0.83
Di-DNP	0.61	0.50	0.60	0.67
Tri-DNP	0.42	0.25	0.40	0.50
Tetra-DNP	0.18	0.0	0.20	0.33
Penta-DNP	0.0	0	0.0	0.17
Hexa-DNP	—	—	—	0.0

Spots on the chromatogram were distributed as follows:

Amt. of gramicidin S in the mixture	Amt. of DNFB added	Distance of spots from the origin, cm (movement toward the cathode)*		
10	1.5	0.0	2.6	5.3
10	3.0	0.0	2.6	
10	6.0	0.0		

*Under the conditions employed, gramicidin S migrated 5.3 cm toward the cathode.

The observed and calculated (for two NH_2 groups) relative mobilities of gramicidin S and its DNP-derivatives are as follows:

Derivatives	Observed	Calculated
Gramicidin S	1.0	1.0
Mono-DNP-derivative	0.45	0.50
Di-DNP-derivative	0.0	0.0

the presence of the resin ÉDÉ-10P, which was used to neutralize the acid formed. Composition of the reaction mixtures was adjusted so that one equivalent of antibiotic was reacted with amounts of DNFB ranging from 1/8 to 3 mmoles. It is unnecessary to know the exact equivalent weight of the antibiotic, since the range of concentrations of DNFB is sufficiently wide to insure the formation of all possible derivatives even if the equivalent weight were two to three times lower or higher. The reaction period chosen was fairly prolonged so that practically all the DNFB was utilized.

Mixtures in which amounts of DNFB and polymyxin M were approximately equivalent contained considerable amounts of higher substitution products and completely substituted dinitrophenyl-polymyxin M. Mixtures in which polymyxin was in significant excess were richer in lower substitution products. By varying the composition of the mixtures it is easy to select optimal ratios of reactants at which approximately equal amounts of the various substitution products are formed.

Triethylamine may be used as a neutralizing agent during dinitrophenylation. It was shown, however, that the presence of even small amounts of its salts hinders markedly the electrophoretic separation of components of the mixture. The separation of DNP-derivatives obtained using an anion-exchange resin as a neutralizing agent proceeds without hindrance, with clearly visible round spots being formed. It should be noted that complete binding of anions by the resin is not obligatory, since even with partial binding all

TABLE 4 Relative Electrophoretic Mobilities of Partially Substituted Derivatives of Polymyxin M

Derivative	Observed		Calculated for		
	DNP-derivatives	Carbobenzoxy derivatives	4 NH_2-groups	5 NH_2-groups	6 NH_2-groups
Unsubstituted	—	—	1,0	1,0	1,0
Monosubstituted	0,79	0,81	0,75	0,80	0,83
Disubstituted	0,59	0,63	0,50	0,60	0,67
Trisubstituted	0,40	0,40	0,25	0,40	0,50
Tetrasubstituted	0,19	0,19	0,0	0,20	0,33
Pentasubstituted	0,0	0,0	—	0,0	0,17
Hexasubstituted	—	—	—	—	0,0

possible substitution products will be obtained. The utilization of exchange resins as neutralizing agents during the dinitrophenylation reaction has not been previously described.

It was also found that the method of partial dinitrophenylation may be simplified still further if the reaction is carried out with the free base of the antibiotic. During the reaction of free amino groups of polymyxin M with DNFB, hydrofluoric acid is formed and is immediately bound by the amino groups which have not yet entered into the reaction. Since such amino groups do not react with DNFB, the reaction gradually slows down and finally comes to a halt. Theoretically, it shoud cease when half the amino groups are substituted, and it is just this level of substitution which is most suitable for detection of the maximum number of reaction products. Thus, this variant of the procedure automatically insures a sufficient degree of substitution.

It is unnecessary to isolate the free base of the antibiotic in pure form before subjecting it to the partial substitution reaction. In the reaction we used a solution obtained by treatment of a polymyxin M sulfate solution with the resin ÉDÉ-10P in the OH form. Conversion of the salt of the antibiotic to the free base need not be complete, moreover, since the only effect of incomplete conversion is to increase somewhat the amount of lower substitution products. The quantity of DNFB used in the reaction with the free base may be altered within wide limits inasmuch as the process is self-regulating and an excess of DNFB cannot substantially affect the extent of subsitution. It has been shown that even with a threefold excess all possible substitution products are formed in fairly large amounts.

The properties of the dinitrophenylation reaction in the absence of a neutralizing agent, which have been described, are such that it is possible to carry out the reaction directly on paper with microquantities of the free base (25-50 μg). Electrophoretic separation of the reaction products is as effective as with large amounts of the polymyxin.

In selecting the conditions of electrophoretic separation it is necessary to take into consideration the special difficulties arising because of the poor solubility of completely substituted DNP-polymyxin M in a majority of solvents. Evidently the higher DNP-derivatives of polymyxin M are also poorly soluble in buffer solutions used in electrophoresis and their movement toward the cathode is thereby impeded, notwithstanding the presence of some amino groups. This accounts for the fact that with 30% acetic acid as the electrolyte we found only three migrating derivatives, the mono-, di-, and tri-dinitrophenyl-polymyxins. It was not possible to locate a clearly defined spot of tetra-DNP-polymyxin M under these conditions. After trying several solvent systems, we settled on a formic acid – acetic acid – water mixture, in which the DNP-derivatives of polymyxin M are quite soluble.

Electrophoretic separation in this system yields four clearly visible spots migrating toward the cathode. These correspond to the mono-, di-, tri-, and tetra-DNP-polymyxin M derivatives. In addition, one derivative remains at the origin. It contains no amino groups, being the penta-DNP- polymyxin M derivative. On the basis of these data it may be concluded that polymyxin M has five free amino groups.

This conclusion is confirmed by inspection of the values for relative electrophoretic mobilities of the partially substituted derivatives of polymyxin M (Table 4).

If polymyxin M has five amino groups, its monosubstituted derivative then possesses four free amino groups. Since the mobility of these compounds is determined chiefly by the number of free amino groups, the monosubstituted derivative should migrate at a rate 4/5 that of the free polymyxin M. (Experiment shows that the increase in molecular weight with the uptake of a dinitrophenyl group may be neglected). Mobilities of other derivatives may be calculated on the same basis. The data of Table 4 show that experimentally determined mobilities are close to calculated values. It should be borne in mind that for accurate determination of relative mobilities electrophoresis should be carried out at the smallest possible potential gradient with a minimum amount of migration, since in the neighborhood of the cathode mobility declines perceptibly.

In determining the number of free amino groups in polymyxin M we also utilized the reaction of hydro-

genolytic decomposition of carbobenzoxy-polymyxin M. As is well known, the carbobenzoxy group is easily removed from amino compounds by catalytic hydrogenation. If hydrogenolysis is incomplete, a mixture of partially substituted derivatives should be formed. The number of carbobenzoxy derivatives in such a mixture corresponds to the number of free amino groups in the polymyxin molecule.

Penta-carbobenzoxy-polymyxin M dissolved in formic acid was hydrogenated in the presence of a palladium catalyst. At intervals samples were withdrawn and subjected to electrophoresis. It was established that five partially substituted carbobenzoxy derivatives are formed in this process. The agreement of observed mobilities with calculated mobilities is in this case poorer than with dinitrophenyl derivatives. This is probably due to the fact that here the potential gradient was three times greater. However, the relative mobilities of the carbobenzoxy derivatives form a series of regularly decreasing values (from free polymyxin to penta-carbobenzoxy-polymyxin) without any gaps.

It should be noted that after removal of the carbobenzoxy groups the antibiotic activity is restored.

Data obtained by this method once more confirm the finding that there are five free amino groups in polymyxin M. Since the equivalent weight of this compound is 237 ±5, the molecular weight is 1185 ± 25 (free base). This value is in good agreement with the minimal value calculated from the amino acid composition (1159).

A comparison of the number of free amino groups, five, with the number of α, γ-diaminobutyric acid residues in the molecule, six, shows that one residue is masked and has no free amino groups. In full agreement with this is our finding that in hydrolyzates of DNP-polymyxin M and of deaminated polymyxin M there is always present unsubstituted α, γ-diaminobutyric acid, which evidently occupies a "corner" position in the molecule, being acylated in both amino groups.

To check the methods of determining the number of free amino groups and the molecular weight of polymyxin M, we used them with polymyxin B and gramicidin S, which are fairly well investigated compounds. Partial dinitrophenylation of polymyxin B proceeds in a manner similar to that with polymyxin M. Reaction products formed are the mono-, di-, tri-, tetra-, and pentasubstituted derivatives. In this way it was found that there are five free amino groups and that the molecular weight is 1260 ± 50, which is in agreement with published information. From the structural formula for polymyxin B [11], the molecular weight is 1220. Hausmann and Craig [5], using the partial dinitrophenylation method, obtained a molecular weight for polymyxin B of 1150 ± 10%. (Dinitrophenyl derivatives were separated by countercurrent distribution).

With partial dinitrophenylation of gramicidin S the mono- and disubstituted derivatives are formed. The calculated molecular weight for this compound is 1108 ± 10. The molecular weight calculated from the structural formula is 1140.

SUMMARY

A study was made of the method of determining the number of free amino groups and the molecular weight of antibiotics by partial substitution and subsequent separation of the reaction products by paper electrophoresis. It was shown that partial dinitrophenylation of compounds containing amino groups may be carried out directly on paper. This was the basis of a micromethod of determining free amino groups in the antibotic molecule.

It was established that polymyxin M has five free amino groups and that its molecular weight is 1185 ± 25. A check of the methods developed in our laboratory using polymyxin B and gramicidin S showed them to be quite accurate, since they yield values in good agreement with published data.

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THE ISOLATION AND PURIFICATION OF GLUTAMINE TRANSAMINASE FROM RAT LIVER*

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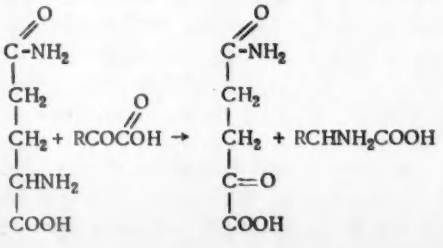
In 1947-1948, Greenstein and co-workers [1-2] announced the presence in rat liver of two enzyme systems for deamidation of glutamine, one of which was activated by phosphate, arsenate and sulfate and was termed glutaminase I; the second was activated by α- keto acids and was termed glutaminase II [3-7]. Separation of these two enzyme systems was achieved by Errera [8] in 1949.

In 1950, Meister and Tice were first to report on enzymatic transamination between glutamine and a number of α-keto acids with the subsequent formation of α- ketoglutarate, ammonia, and the corresponding amino acid [9]; the reaction was catalyzed by a liver anzyme preparation. Meister proposed that the intermediate product in the combined reactions of transamination and deamidation of glutamine was the α-monoamide of α-ketoglutaric acid—a product of enzymatic α-deamidation of glutamine [10].

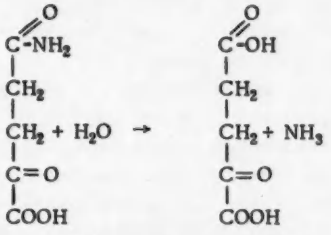
Subsequently, Meister and co-workers [11] described an analogous liver enzyme system, which specifically catalyzed the combined reaction of transamination and deamidation of asparagine in the presence of various α-keto acids. In 1953 Meister was able to obtain a purified amidase, hydrolyzing ω-amides of ketoglutaric and oxaloacetic acids (ketoamidase), which did not contain glutamine transaminase.

According to the presentations of Meister the combined reactions of transamination and deamidation went through the following two stages:

1. Transamination



2. Deamidation



The first reaction is catalyzed by glutamine transaminase; the second, by ketoamidase (ω-amidase).

Meister assumed that pyridoxal phosphate did not act as a coenzyme in the combined enzymatic reaction of transamination and deamidation. Olenicheva (1955, [12]), in the author's laboratory, observed that the combined reactions of transamination and deamidation of glutamine and asparagine were considerably weakened in livers of rats maintained on a diet deficient in vitamin B_6. In in vitro experiments the activating action of pyridoxal phosphate was established. Olenicheva has likewise observed combined reactions of transamination and deamidation in plants.

Meister purified ketoamidase from transaminase, but was not successful in obtaining glutamine and asparagine transaminases free from ketoamidase and from some other transaminases [10-13]. The present authors selected as their problem the isolation and purification of glutamine transaminase (GL-NH_2-transaminase), in order to study its its enzymatic properties.

* Abbreviations used in the text: GL) glutamic acid; AS) asparaginic acid; GL-NH_2) glutamine; AS-NH_2) asparagine; OAC) oxaloacetic acid; AL) alanine; PY) pyruvic acid; PHP) phenylpyruvic acid; α-KG) α-ketoglutaric acid; AL-transaminase) alanine-glutamate-transaminase; AS-transaminase) aspartate-glutamate-transaminase; GL-NH_2-transaminase) glutamine transaminase; AS-NH_2-transaminase) asparagine transaminase; VB) veronal buffer; DEAE-cellulose) diethylaminoethyl-cellulose.

EXPERIMENTAL

Determination of enzyme activity.

In the early purification stages, when the preparation contained glutaminase I and ketoamidase, the GL-NH_2-transaminase activity was determined by the difference in values of increase of NH_3 in samples containing glutamine and a keto acid and samples incubated with glutamine alone.

In much later purification stages, when in the enzyme preparation practically no glutaminase was present but there was still ketoamidase, the GL-NH_2-transaminase activity was determined by the increase in values of phenylalanine and ammonia; in this case the quantity of ammonia formed was equivalent to the quantity of phenylalanine formed.

During the last purification stages, when the preparations contained no glutaminase or ketoamidase, the GL-NH_2-transaminase activity was determined by the quantity of phenylalanine formed.

The experimental samples (total volume, 2 ml) contained 14 μmoles of glutamine and 14 μmoles of Na-phenylpyruvate in 0.025 molar veronal buffer (pH 8.4); duration of incubation was two hours. The unit of transaminase activity was taken as the amount of amino acid in μmoles formed under the described conditions. Specific enzyme activity (sp. act.) was expressed as the number of units calculated per one milligram of protein; ammonia was determined by the method of Speck [14]; phenylalanine, by the paper chromatographic method of Giri [15], and protein, by the method of Lowry [16]. For the purposes of determining the activity of enzyme impurities (AL-transaminase, AS-transaminase, phenylalanine-glutamate-transaminase), the corresponding amino acids (AL, GL, phenylalanine) were quantitatively determined by paper chromatography [15]. In some experiments, the activities of AL-transaminase and AS-transaminase were determined enzymatically using the SGPT and SGOT tests of the Boehringer firm (designed for the determination of the indicated transaminases in blood). Enzyme activities were expressed in μmoles of OAC or PY formed under standard conditions in 1 min. The optical density of the samples was measured at 340 mμ in an SF-4 spectrophotometer; a decrease in optical density (ΔE_{340}) of 0.1 corresponded to the disappearance of 0.0161 μmoles DPNH and the formation (via transamination) of 0.0161 μmoles of OAC or of PY.

The course of purification of glutamine transaminase.

After a number of preliminary investigations, the authors settled on utilization of the following operations for the purpose of obtaining pure GL-NH_2-transaminase.

1. Production of the rat liver homogenate and extract. Cooled liver tissue from well-fed mature male rats was homogenized for 2 min with a 3-fold volume of 0.05 M veronal buffer (VB), pH 8.0. The resulting homogenate was centrifuged for one hour at 8000 rpm and the precipitate, containing a large amount of glutaminase, was discarded.

2. Heating. According to the data of Errera and Greenstein, glutaminase I is not heat-stable, whereas glutaminase II (i.e., the transaminase-ketoamidase system) is stable. On the basis of this, the authors utilized heating for the purpose of purifying GL-NH_2-transaminase.

The extract was heated at 50° for 12 min. During the heating there was a copious precipitation of denatured protein. The solution was cooled rapidly and the precipitate was removed by centrifugation. The extract obtained contained very little glutaminase I, and thus during this stage of enzyme purification there was equivalent formation of phenylalanine and of ammonia.

3. Precipitation with ammonium sulfate. The extract remaining after removal of the protein after heating was dialyzed against tap water for 10 hrs. After dialysis a precipitate appeared which was removed by centrifugation. Ammonium sulfate was added to the supernatant to 30% of saturation. The inactive precipitate was removed by centrifugation; an active fraction was precipitated from the supernatant by addition of $(NH_4)_2SO_4$ to 60% of saturation. The precipitate was dissolved in a small quantity of water. The protein solution was dialyzed against tap water overnight at 4-6° and was then dried by lyophilization.

4. First electrophoresis. For further purification of the enzyme, we utilized electrophoresis in vertical columns filled with cellulose powder [17]. This method, which is widely utilized at present for protein and enzyme fractionation, is noted for its high resolving ability. Electrophoresis was conducted in 0.05 M VB (pH 8.4) with a voltage of 3-4 v/cm and a current strength of 40-45 ma. To the column, which was 2 × 90 cm, 4 ml of the solution was added, containing approximately 300 mg of protein. Duration of electrophoresis was 37-38 hrs.

The proteins of the starting preparation moved toward the anode (i.e., the lower end of the column). At the end of the experiment the column was eluted with VB, and 3-ml fractions were collected with an automatic collector. In the fractions obtained we determined the protein content and the activity of the various transaminases (spectrophotometrically; extinction at 280 mμ). In the active fractions, the protein content was ascertained by the method of Lowry [16].

Figure 1 shows the distribution of protein and transaminase specific activity (sp. act.) in the obtained fractions. GL-NH_2-transaminase activity was observed in fractions 28-44; AS-NH_2-transaminase activity, in fractions 54-72; phenylalanine-glutamate-transaminase, in fractions 78-88. All of these fractions were distinctly separated from one another. In fractions containing GL-NH_2-transaminase, AL-transaminase was observed in large amounts and AS-transaminase in a small amount; in the same fractions there was complete absence of

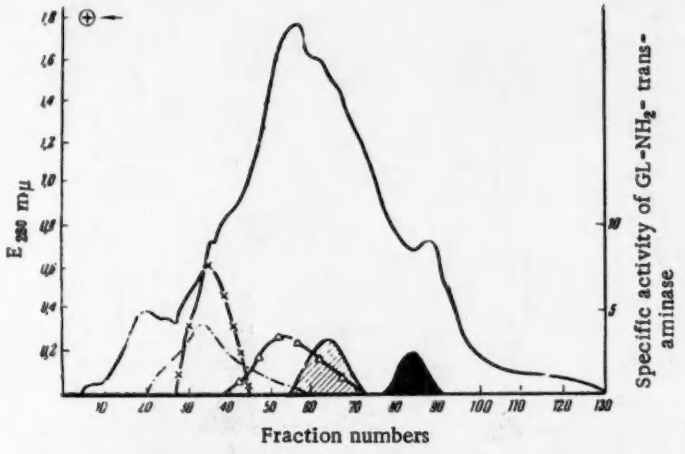


Fig. 1. Distribution of protein and specific transaminase activity during electrophoresis in vertical column with cellulose powder. Solide line – protein; line with crosses – GL-NH_2-transaminase; line with dots – AL-transaminase; line with triangles – AS-transaminase; the shaded peak – AS-NH_2-transaminase; black peak – phenylalanine-glutamateltransaminase.

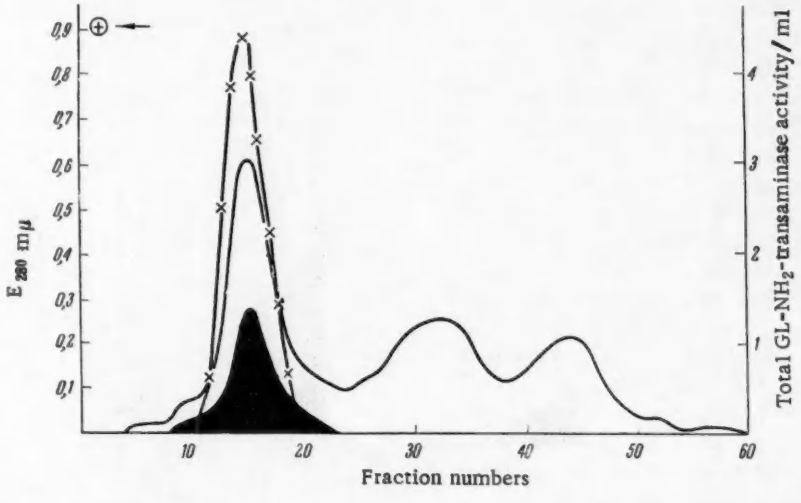


Fig. 2. Distribution of protein and total GL-NH_2-transaminase activity after repeated electrophoresis in a vertical cellulose powder column. Solid line – protein; line with crosses – GL-NH_2-transaminase; black peak – AL-transaminase.

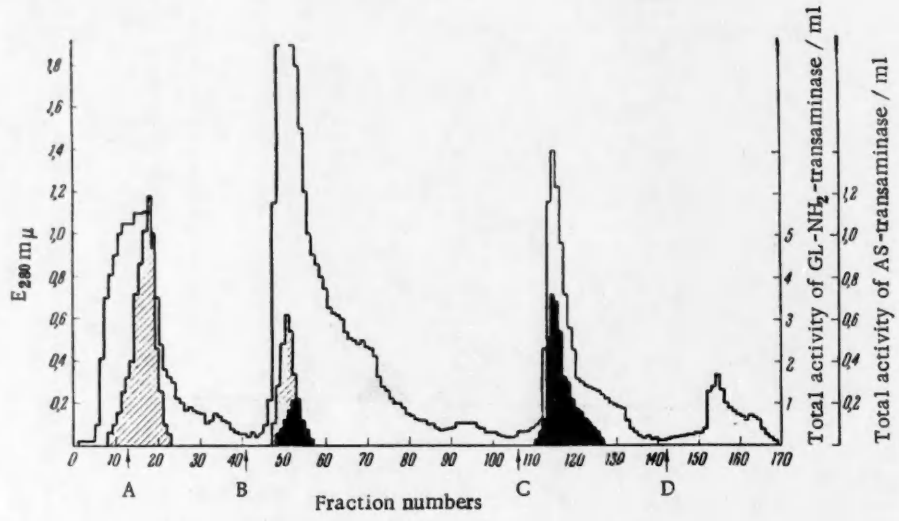


Fig. 3. Distribution of protein and AS-transaminase and GL-NH_2-transaminase total activities during fractionation on DEAE-cellulose. Solid line – protein; shaded peaks – AS-transaminase; black peaks – GL-NH_2-transaminase; A – 0.025 M VB, pH 8.4; B, C, D, respectively – 0.1, 0.2, and 0.3 M NaCl in 0.025 M VB, pH 8.4.

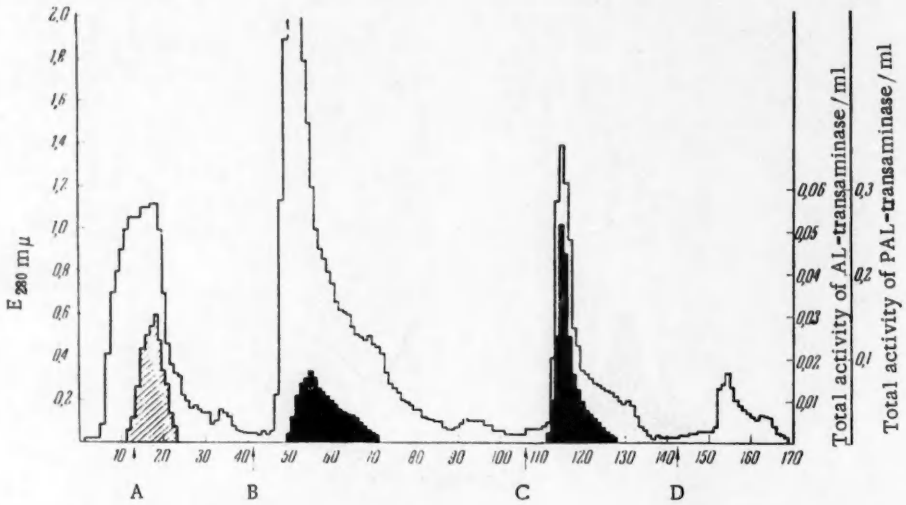


Fig. 4. Distribution of protein and AL-transaminase and phenylalanine-glutamate-transaminase total activities during fractionation on DEAE-cellulose. Solid line – protein; shaded peak – phenylalanine-glutamate-transaminase; black peaks – AL-transaminase.

TABLE 2. Purification of GL-NH_2-Transaminase Via Adsorption on DEAE-Cellulose and Subsequent Zone Electrophoresis.

Purification stage	Total activity M	Protein, mg	Sp. act.	Yield, %
Fraction precipitated within 0.3-0.6 saturation limits of $(NH_4)_2SO_4$	180	240	0,75	(100)
Separation on DEAE-cellulose	115	32	3,6	64
Electrophoresis	85,3	10,4	8,2	47,5

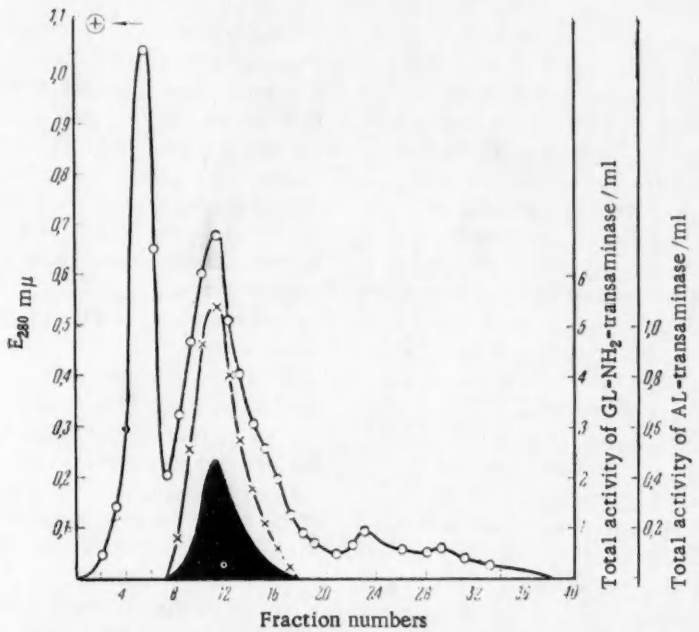


Fig. 5. Distribution of protein and GL-NH_2-transaminase and AL-transaminase total activities after electrophoresis in vertical cellulose powder columns. Solid line – protein; line with crosses – GL-NH_2-transaminase; black peak – AL-transaminase.

The experimental results are presented in Table 2 and show that with the help of this operation the sp. act. of GL-NH_2-transaminase was increased 2.3-fold as compared with the fraction obtained after DEAE-cellulose, and 75-fold as compared to the sp. act. of the starting homogenate.

Electrophoretic mobility of glutamine-transaminase and of alanine-glutamate-transaminase.

For the purpose of determining the electrophoretic mobility of GL-NH_2-transaminase, the solution of the active protein fraction, which was obtained by single electrophoresis in a starch block in VB, pH 8.4, $\mu = 0.05$, was subjected to analysis in the same buffer in a Tiselius apparatus. During free electrophoresis, we observed three protein fractions with closely situated peaks (Fig. 6). All of the GL-NH_2-transaminase was concentrated in the middle protein peak, possessing (on the ascending border in the Tiselius apparatus) an electrophoretic mobility of $-5.1 \cdot 10^{-5}$ $cm^2 \cdot sec^{-1} \cdot v^{-1}$. AL-transaminase was observed in the same peak. The experiments described show that GL-NH_2-transaminase and AL-transaminase had similar electrophoretic mobilities. The character and distribution of the protein peaks observed in the active fraction by means of free electrophoresis (Fig. 6) and by zone electrophoresis after DEAE-cellulose adsorption (Fig. 5) were quite comparable.†

DISCUSSION

With the help of operations described in the experimental sections it was possible to purify rat liver GL-NH_2-transaminase with a 76-84-fold increase in specific activity of the enzyme; the yield of the purified enzyme was 13%.

† The authors wish to express their sincere gratitude to V. D. Uspenskaya for her help with experiments on free electrophoresis of enzyme preparations.

TABLE 1. Purification of Glutamine Transaminase From Rat Liver

Purification stage	Total act., μM	Protein, mg	Sp. act.	Yield, %
Homogenate	582,4	5300	0,11	(100)
Heated extract	292	885	0,33	50
Fraction precipitated within 0.3-0.6 saturation limits of $(NH_4)_2SO_4$	207,2	305	0,68	35,7
First electrophoresis	140	25	5,6	24
Second electrophoresis	74,4	9	8,4	12,8

glutaminase, ketoamidase, AS-NH_2-transaminase, and phenylalanine-glutamate-transaminase. At this stage of purification the sp. act. of GL-NH_2-transaminase was elevated 50-fold as compared to the starting homogenate.

5. Repeated column electrophoresis. For further purification of GL-NH_2-transaminase, the most active fractions obtained after the first electrophoresis were dialyzed against distilled water and concentrated to a small volume in a cellophane bag with an air current at 5-8°. The concentrated active-fraction solution was subjected to repeated electrophoresis in a column with dimensions of 1.3 × 60 cm. Electrophoresis was conducted for 34 hrs in 0.05 M VB (pH 8.4) with a voltage of 3-4 v/cm and a current strength of 13-15 ma.

Figure 2 shows the distribution of protein and total activities (tot. act.) of GL-NH_2-transaminase and AL-transaminase in fractions obtained after the second electrophoresis. As is evident from this figure, both activities were concentrated in one protein peak. With purification, the activity of both transaminases increased in parallel. At this stage the sp. act. of GL-NH_2-transaminase was increased 76-84-fold over the original (Table 1).

During all of the purification stages, GL-NH_2-transaminase activity was well preserved during dialysis (with prolonged dialysis against water, a small portion of the active protein came out of the solution with the globulin fraction). Preincubation of the experimental samples for 10 min with pyridoxal phosphate (5γ/ml) did not result in any essential increase in GL-NH_2-transaminase activity of the purified undialyzed and dialyzed enzyme preparations. This pointed to the firmness of the bond between the apoenzyme and pyridoxal phosphate.

Purification of GL-NH_2-transaminase by adsorption on DEAE-cellulose and subsequent zone electrophoresis.

During the course of the study, the authors attempted to utilize DEAE-cellulose anionite for purification of GL-NH_2-transaminase; this has been employed during the past few years for isolation and purification of enzymes and other proteins [18].

The chloride form of the anionite was used; the hydroxyl form of DEAE-cellulose was placed on a column (3 × 6 cm) and it was transferred into the chloride form by washing with 2M NaCl in 0.025 M VB (pH 8.4) until the filtrate gave a neutral reaction [19]; then the column was washed with VB. In the experiment a dialyzed enzyme preparation obtained after precipitation with $(NH_4)_2SO_4$ was used. Into a column prepared as described above there was placed 240 mg of protein in 30 ml of 0.025 M VB. Elution was based on a stepwise increase in the solution ionic strength; namely, VB and solutions of VB containing 0.1, 0.2 and 0.3 M NaCl were utilized sequentially.

Figures 3 and 4 show the distributions of protein and tot. act. of several transaminases in fractions obtained in the given experiment.

As is evident from the figures, AS-transaminase was observed in two protein fractions: The major portion was in the fraction removed with 0.025 M VB (pH 8.4) and a small portion, in the fraction removed by buffer containing 0.1 M NaCl. Phenylalanine-glutamate-transaminase was observed in one protein peak which was removed by the buffer, i.e., in the same fraction as the major mass of AS-transaminase. In the protein peak removed with the 0.1 M concentration of NaCl AS-NH_2-transaminase was also found. Activities of AL-transaminase and of GL-NH_2-transaminase were found in two protein fractions; the major portion of both activities was in fractions removed with 0.2 M NaCl, and a very small portion was in the fractions removed with 0.1 M NaCl. In comparison with the starting fraction, the sp. act. of GL-NH_2-transaminase, as the result of adsorption on DEAE-cellulose, was increased 4.8 times; the yield of the enzyme during this stage comprised 64% (Table 2).

GL-NH_2-transaminase obtained during this stage was subjected to further purification by zone electrophoresis in a vertical cellulose powder column with dimensions of 1.3 × 60 cm. Electrophoresis was conducted for about 30 hrs under the above-described conditions. Before electrophoresis the active GL-NH_2-transaminase fraction was dialyzed against distilled water and 10^{-4} M unithiol; it was then concentrated to a small volume at 4-6°.

Figure 5 shows the distributions of protein and total activities of GL-NH_2- and AL-transaminases in fractions obtained after electrophoresis. As is evident from this figure, both activities were concentrated in one protein peak and increased during the course of purification. The GL-NH_2-transaminase fraction, obtained after adsorption and zone electrophoresis, did not contain glutaminase, keto amidase, AS-transaminase, phenylalanine-glutamate-transaminase, or AS-NH_2-transaminase.

The purified enzyme was quite stable; it did not dissociate into apo- and coenzyme during treatments or prolonged dialysis. Maximally purified preparations during free and zone electrophoresis and during adsorption on cellulose anionite behaved as a homogeneous protein with an electrophoretic mobility of $-5.1 \cdot 10^{-5}$ cm^2 · sec^{-1} · v^{-1}. The specific activity of the most highly purified preparations was relatively small in comparison with a number of other transaminases, i.e., ~8.4 micromoles/mg/2 hrs. Beginning with the first purification stages (heating, fractionation with ammonium sulfate), the preparations were freed from glutaminase; during further purification, GL-NH_2-transaminase became completely free from keto amidase, which accompanied it in preparations described by Meister and co-workers [10, 13], and also free from a number of transaminases (AS-, AS-NH_2, phenylalanine-glutamate-transaminase).

However, as is evident from the presented experimental material, during all of the processing stages, during utilization of the various purification methods (salting out, heating, adsorption on DEAE-cellulose, zone and free electrophoresis, and other methods) there was parallel enrichment of both GL-NH_2- and AL-transaminases, whereupon the distribution of activities of both transamination reactions according to adjacent protein fractions was closely similar (Fig. 1-5). From this, it follows that the reactions mentioned are either catalyzed by the same enzyme, or else this is accomplished by two enzymes possessing quite similar physicochemical properties and identical electrophoretic mobilities.

In order to better solve the question on the identity or nonidentity of liver GL-NH_2- and AL-transaminases, the authors explored the influence of the antibiotic cycloserine on the activity of the described transaminases. According to data from the authors' laboratory [20], AL-transaminase differs from a number of other transaminases by its especially high susceptibility to the inhibitory action of DL-cycloserine [21]. In experiments with a GL-NH_2-transaminase preparation (active fraction after single electrophoresis and adsorption on DEAE- cellulose) it was observed that in this preparation DL-cycloserine (10^{-4} M) inhibited 90-95% of AL-transaminase activity, while at the same time and under the same conditions the GL-NH_2-transaminase activity was inhibited only by 15%. This seems to indicate the presence in the preparation of two enzymes of similar physicochemical behavior, but differing in substrate specificity and in their relation toward the inhibitory action of cycloserine.

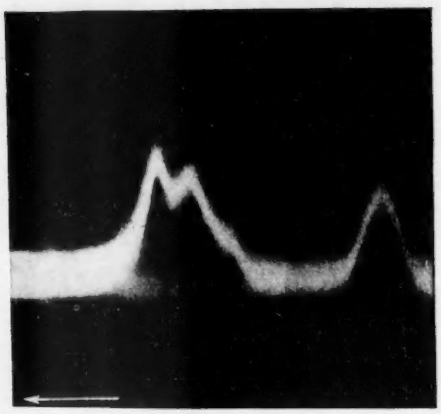


Fig. 6. Electrophoretic distribution of GL-NH_2-transaminase preparation in apparatus of Tiselius. Veronal buffer, pH 8.4, μ=0.05. Ascending boundary during 120 min. The GL-NH_2-transaminase preparation was obtained after starch electrophoresis.

Supporting the view that AL-transaminase and GL-NH_2-transaminase are not identical, data [9,12] are available concerning the fact that the latter enzyme is basically concentrated in the soluble fraction of liver cytoplasm, whereas AL-transaminase is also active in the mitochondrial fraction [22]; also supporting this is the fact that GL-NH_2-transaminase is not found in muscles [11] which contain active AL-transaminase.

The authors' data do not exclude the possibility of the existence of various enzymes performing the reaction AL ⇌ ⇌GL, namely, of specific AL-transaminases (in muscle and possibly liver mitochondria) and of a "bifunctional" enzyme, combining the activities of GL-NH_2- and AL-transaminase in the soluble liver protein fraction. For a well-defined solution of the question concerning the limits of GL-NH_2-transaminase specificity, futher investigations are needed.

SUMMARY

A purified preparation of rat liver GL-NH_2-transaminase was obtained; the specific activity of the enzyme was increased 76-84-fold in comparison with the starting homogenate.

The preparation obtained was completely free from glutaminase and keto amidase as well as from AS-transaminase, AS-NH_2-transaminase and phenylalanine-glutamate-transaminase.

The electrophoretic mobility of glutamine transaminase was determined in veronal buffer, pH 8.4 (μ = =0.05); it was equal (on the ascending boundary) to $-5.1 \cdot \cdot 10^{-5} \cdot$ cm^2 · sec^{-1}. $^{-1}$.

GL-NH_2-transaminase, purified by various means, was accompanied by active AL-transaminase. In their physicochemical properties these two enzymes were evidently very close to one another; their electrophoretic mobilities were practically identical. However, 10^{-4} M DL-cycloserine, which almost completely supressed transamination between glutamic acid and pyruvate, inhibited glutamine transaminase activity only to an insignificant degree (by 15%).

A purified AS-NH_2-transaminase preparation was obtained. It did not contain glutamine transaminase (Fig. 1).

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‡Original Russian pagination. See C. B. translation.

IN THE ALL-UNION BIOCHEMICAL SOCIETY

E.V. Budnitzkaya

Translated from Biokhimiya, Vol. 25, No. 4, p. 768, July-August, 1960

An alternate meeting of the Presidium of the All-Union Biochemical Society took place in Moscow on the 17th of December. A presentation by S. E. Severin, Corresponding Member of the Academy of Sciences, USSR, was heard. It concerned the Plan of the Society's Work for 1960.

In both presentation and discussion, attention was directed toward the necessity of intensifying the work of the Society's branches and the expediency of adopting more effective measures on organization of scientific meetings. In part, it was suggested that the presentations of directors of scientific-research and other institutions should be heard during the meeting of the Society's Council. It was suggested that the editors of biochemical journals be asked to publish, without delay, the works presented during the meetings of the society. Attention was directed to the activity of the Society in connection with the preparations for the Fifth International Biochemical Congress. It was underlined that the Society, a major organizer of the Congress, should reflect plans of preparation to the Congress in their working plans for 1960. Reports for the Fifth International Biochemical Congress should be chosen during the scientific sessions of the Society in 1960. This selection of reports would assist in livening the work of the Society.

In the discussion it was noted that the level of biochemical science must be raised at the earliest possible time. This is necessary and can be accomplished by improving the working conditions in institutions of biochemistry, finding resources for new equipment, producing necessary reagents, and by erecting specialized buildings. It was suggested, in accord with the resolution of the Plenum of the Central Council of the All-Union Biochemical Society, to form a number of commissions for the realization of the accepted resolutions.

In connection with this, the Presidium of the Central Council of the All-Union Biochemical Society approved the personnel of the following commissions:

1. Commisions on conducting the All-Union conference concerning the question of preparing specialists in biochemistry: S. R. Mardashov—chairman; A. N. Belozerskii, G. Kh. Bunatyan, G. E. Vladimirov, Z. S. Gershenovich, V. G. Klimenko, P. A. Kometiani, Yu. N. Kremer, V. L. Kretovich, M. F. Merezhinskii, A. A. Shmidt, V. V. Yurkevich.
2. Commission concerning questions of nomenclature: A. E. Braunshtein—chairman; V. N. Bukin, A. R. Guseva, A. V. Kotel'nikova, A. A. Krasnovskii, V. L. Lyubimov, O. L. Polyanovskii, E. L. Rozenfel'd, B. G. Savinov, I. V. Torgov, A. S. Khokhlov, V. S. Shapot Tzukerman, N. A. Yudaev.
3. Commission on organization of consulting work in the branches and sections of the Society: S. Ya. Kaplanskii—chairman; A. Z. Babaev, A.S. Vecher, G. Ya. Gorodiskaya, N. N. D'yachkov, and others.
4. Commission on the questions of publications: B. N. Stepanenko—chairman, V. S. Asatiani, N. N. Demin, V. S. I'in, É. É. Martinson, O. A. Pavlinova, T. S. Paskhina, V. I. Rosengardt, Ya. Kh. Turakulov, R. V. Chagovets, L. S. Cherkasova.

The plan for conducting conferences in 1960 of the Belorussian, the Latvian and Estonian, the Armenian and Georgian, and the Moscow Biochemical Societies was also approved.

The composition of the International Union of Biochemists was discussed and the council personnel of the newly organized sections was approved.

The work of sending out the Society membership cards and materials on the Fifth International Biochemical Congress is presently in the hands of the Secretariat of the Central Council of the All-Union Biochemical Society.

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

*BRITISH-AMERICAN TRANSLITERATION SYSTEM.

(continued)

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
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Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
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